Universidade de Aveiro Departamento de Química
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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica do Dr. Pedro Miguel Dimas Neves Domingues, Professor Auxiliar do Departamento de Química da Universidade de Aveiro, e do Dr. Francisco Manuel Lemos Amado, Professor Associado do Departamento de Química da Universidade de Aveiro

Dedico este trabalho à memória do meu pai.

o júri

presidente

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palavras-chave Oxidação de proteínas, Glicosilação não-enzimática, Espectrometria de massa

resumo

A glicosilação não-enzimática e o stress oxidativo representam dois processos importantes visto desempenharem um papel importante no que respeita às complicações de vários processos patofisiológicos. No presente, a associação entre a glicosilação não-enzimática e a oxidação de proteínas é reconhecida como sendo um dos principais responsáveis pela acumulação de proteínas não-funcionais que, por sua vez, promove uma contínua sensibilização para um aumento do stress oxidativo ao nível celular. Embora esteja disponível bastante informação no que respeita aos dois processos e suas consequências ao nível estrutural e funcional, permanecem questões por esclarecer acerca do que se desenvolve ao nível molecular. Com o objectivo de contribuir para uma melhor compreensão da relação entre a glicosilação não-enzimática e a oxidação, proteínas modelo (albumina, insulina e histonas H2B e H1) foram submetidas a sistemas *in vitro* de glicosilação não-enzimática e oxidação em condições controladas e durante um período de tempo específico. A identificação dos locais de glicosilação e oxidação foi realizada através de uma abordagem proteómica, na qual após digestão enzimática se procedeu à análise por cromatografia líquida acoplada a espectrometria de massa tandem (MALDI-TOF/TOF).

Esta abordagem permitiu a obtenção de elevadas taxas de cobertura das sequências proteicas, permitindo a identificação dos locais preferenciais de glicosilação e oxidação nas diferentes proteínas estudadas. Como esperado, os resíduos de lisina foram os preferencialmente glicosilados. No que respeita à oxidação, além das modificações envolvendo hidroxilações e adições de oxigénio, foram identificadas deamidações, carbamilações e conversões oxidativas específicas de vários aminoácidos. No geral, os resíduos mais afectados pela oxidação foram os resíduos de cisteína, metionina, triptofano, tirosina, prolina, lisina e fenilalanina. Ao longo do período de tempo estudado, os resultados indicaram que a oxidação teve início em zonas expostas da proteína e/ou localizadas na vizinhança de resíduos de cisteína e metionina, ao invés de exibir um comportamente aleatório, ocorrendo de uma forma não-linear por sua vez dependente da estabilidade conformacional da proteína. O estudo ao longo do tempo mostrou igualmente que, no caso das proteínas pré-glicosiladas, a oxidação das mesmas ocorreu de forma mais rápida e acentuada, sugerindo que as alterações estruturais induzidas pela glicosilação promovem um estado pro-oxidativo. No caso das proteínas pré-glicosiladas e oxidadas, foi identificado um maior número de modificações oxidativas assim como de resíduos modificados na vizinhança de resíduos glicosilados. Com esta abordagem é realizada uma importante contribuição na investigação das consequências do dano 'glico-oxidativo' em proteínas ao nível molecular através da combinação da espectrometria de massa e da bioinformática.

keywords Protein oxidation, Glycation, Mass spectrometry

abstract

Glycation and oxidative stress are two important processes known to play a key role in complications of many pathophysiological processes. It is nowadays acknowledged the association between glycation and oxidation events as a major responsible for the accumulation of non-functional damaged proteins that in turn promote continuous sensitization to further oxidative stress at the cellular level. Despite the large amount of information concerning both events and their consequences at structural and functional levels, questions remain to answer on what happens at the protein molecular level. With the aim of contributing to better understand the interrelationship between glycation and oxidation, model proteins (BSA, insulin and histones H2B and H1) were submitted to *in vitro* systems of glycation and oxidation under controlled conditions and through a specific period of time. Identification of glycation and oxidation sites was performed through a proteomics approach. Protein samples were enzimatically digested and further analyzed by nano-liquid chromatography coupled to MALDI-TOF/TOF mass spectrometry.

This approach allowed obtaining high protein coverage rates, enabling the identification of the most susceptible sites of glycation and oxidation in the different studied proteins. As expected, lysine residues were preferentially glycated and with respect to oxidation, besides protein hydroxyl derivatives and oxygen additions, modifications such as deamidations, carbamylations and specific amino acid oxidative conversions were detected. In general, the main affected amino acids by oxidative damage were cysteine, methionine, tryptophan, tyrosine, proline, lysine and phenylalanine. The time-course study of the oxidative damage indicated the oxidative attack, rather than occurring randomly, initiates at surface-exposed regions and/or near cysteine and methionine residues and occurs in a non-linear way depending on the conformational stability of the protein. Time-course analysis also showed a more pronounced and earlier occurrence of the oxidative damage in the case of preglycated proteins, suggesting that structural changes caused by glycation induce a pro-oxidant state. This increased oxidative damage included not only a greater number of oxidative modifications, but also of oxidized residues, occurring in the vicinity of the glycated residues. Through this kind of approach, an important contribution is made in the investigation of the consequences of protein ‘glycoxidative’ damage at a molecular level through the profit combination of mass spectrometry and bioinformatics.

Index

Abbreviations

3-DG	3-deoxyglucosone
α-CHCA	4-hydoxy-α-cyanocinnamic acid
ACN	acetonitrile
ADP	adenosine diphosphate
AGE	advanced glycation endproduct
APCI	atmospheric pressure chemical ionization
ATP	adenosine triphosphate
BAC	boronate affinity chromatography
BSA	bovine serum albumin
cDNA	complementary DNA
CEL	N^{ε}-(carboxyethyl)lysine
CID	collision-induced dissociation
CMA	(carboxymethyl)arginine
CML	Nε-(Carboxymethyl)lysine
Da	dalton
DMPO	dimethyl-1-pyrroline-N-oxide
DNA	deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DOPA	3,4-dihydroxyphenylalanine
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
ECD	electron capture dissociation
EDTA	Ethylenediamine tetraacetic acid
ELISA	enzyme-linked immuno sorbent assay
ESI	electrospray ionization
ETD	electron transfer dissociation
FAB	fast atom bombardment
FT	fourier transform
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gas chromatography
GODIC	arginine-lysine-glyoxal crosslink

GOLD	glyoxal-lysine dimer
GR	glutathione reductase
GSH	glutathione
H_2O_2	hydrogen peroxide
HClO	hypoclorous acid
HDL	high-density lipoprotein
HOBr	hypobromous acid
HSA	human serum albumin
IEF	isoelectric focusing
IT	ion trap
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
MALDI	matrix assisted laser desorption ionization
MCO	metal-catalyzed oxidation
MDA	malondialdehyde
MeSOX	methionine sulfoxide
MOLD	imidazolium crosslink
mPBA	3-aminophenylboronic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NMR	nuclear magnetic resonance
$NO^{\bullet}$	nitric oxide radical
OD	optical density
$OH^{\bullet}$	hydroxyl radical
PBS	phosphate buffered saline
PTM	posttranslational modification
QIT	quadrupole-ion trap
QqQ	triple quadrupole
Q-TOF	quadrupole-time of flight
RAGE	receptor for advanced glycation endproduct
RIA	radioimmunoassay
$RO^{\bullet}$	alkoxyl radical
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
RP-HPLC	reversed phase high performance liquid chromatography

RS	reactive species
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TOF	time of flight
UV	ultraviolet radiation

List of original publications

1. Guedes S, Vitorino R, Domingues R, Amado F, Domingues P. Oxidation of bovine serum albumin: identification of oxidation products and structural modifications. Rapid Commun Mass Spectrom. 2009, 23: 2307-15.

2. Guedes S, Vitorino R, Domingues MR, Amado F, Domingues P. Mass spectrometry characterization of the glycation sites of bovine insulin by tandem mass spectrometry. J Am Soc Mass Spectrom. 2009, 20: 1319-26.

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1. Introduction

For the past decades, oxidative stress has been a major topic of research within the scientific community. Particularly after the establishment of the free-radical theory of aging by Herman Denham (Harman, 1956; Harman, 1981), numerous studies and research efforts were conducted and contributed greatly to the present knowledge of radical species, oxygen-derived radicals and other oxygen-derived non-radical reactive species. These metabolites are now considered major players in biochemical reactions, cellular responses, and clinical outcomes, acting both as effector molecules and damaging agents (Harman, 1981; Beckman and Ames, 1998; Sen, 2000; Dizdaroglu *et al.*, 2002).

Oxidative stress is formerly known as the result of excessive production of reactive species that overcomes the biological system protective response, resulting in an imbalance between these two events. Nevertheless, these cellular redox reactions are the basis for numerous biochemical pathways and cellular chemistry, biosynthesis, and regulation (Voeikov, 2006; Winterbourn, 2008), besides being of extreme relevance in the understanding of biological oxidation and radical/antioxidant effects. Chemically, every compound including oxygen that is able of accepting electrons is an oxidizing (also called oxidant) agent (Prior and Cao, 1999). In general these pro-oxidants are referred to as reactive oxygen species (ROS) that can be classified into two groups of compounds, radicals and nonradicals, as shown in table 1.1. The radical group contains compounds such as nitric oxide radical ($NO^{\bullet}$), superoxide ion radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$), and one form of singlet oxygen ($^1O_2$). The group of nonradical compounds contains a large variety of substances, some of which are extremely reactive. Among these compounds produced in high concentrations in the living cell are hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3), and O_2. Among the compounds shown in table 1.1, superoxide, peroxide, and the hydroxyl radical are considered the primary ROS and are responsible for the major research on the role of free radicals in biology and medicine (Wu and Cederbaum, 2003).

Table 1.1 - Radical and nonradical oxygen species (Hrbac and Kohen, 2000).

Name	Symbol
Oxygen radicals	
Oxygen (bi-radical)	$O_2^{\bullet\bullet}$
Superoxide ion	$O_2^{\bullet-}$
Hydroxyl	$OH^{\bullet}$
Peroxyl	$ROO^{\bullet}$
Alkoxyl	$RO^{\bullet}$
Nitric oxide	$NO^{\bullet}$
Nonradical oxygen derivatives	
Hydrogen peroxide	H_2O_2
(Organic peroxide)	ROOH
Hypochlorous acid	HOCl
Ozone	O_3
Aldehydes	HCOR
Singlet oxygen	$^1O_2$
Peroxynitrite	ONOOH

Most ROS are short-lived species that react quickly with other molecules. For example, $OH^{\bullet}$ can survive for 10^{-10} sec in biological systems and its reaction rate constants ($m^{-1}s^{-1}$) towards biological components are extremely high (exceeding $10^9\ m^{-1}s^{-1}$). Nevertheless, is the physiological environment, consisting of factors such as pH and the presence of other species, that has a great influence on the half-life of ROS. Moreover, their toxicity is not necessarily correlated with their reactivity. A longer half-life species might imply a higher toxicity since it allows the adequate time to diffuse and reach a sensitive location where it can interact and cause damage. For example, the relatively long half-life of superoxide radicals permits them to move to locations where they can undergo interaction with other molecules; these radicals can be produced in the mitochondrial membrane, diffuse towards the mitochondrial

genome and reduce transition metals bound to the genome (Kohen and Nyska, 2002). In contrast, a highly reactive species with an extremely short life span, like $OH^{\bullet}$, is produced in locations where it causes damage by interacting with its immediate surroundings (Chen and Schopfer, 1999). The high reactivity of radicals and their short life span illustrate the potential toxic effect and difficulties in preventing oxidative damage.

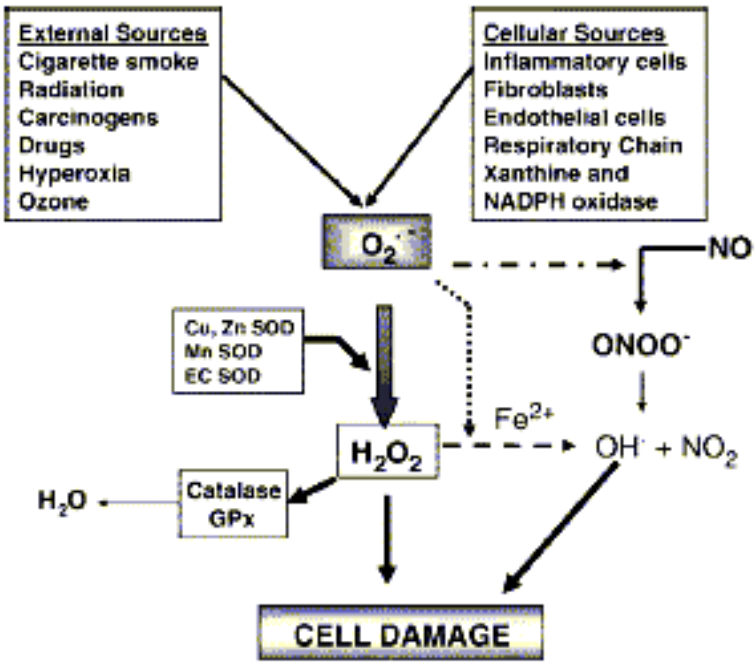

Figure 1.1 – Examples of endogenous and exogenous sources of ROS (Rahman *et al.*, 2006).

Exposure of living systems to the large variety of ROS comes from both exogenous and endogenous sources (Figure 1.1) (Kohen and Gati, 2000; Galli *et al.*, 2005). Endogenous sources include exposure to dioxygen, although being a nonreactive biradical, can independently cause oxidation and damage to proteins and enzymes (Valentine, 1994). Ozone (O_3) is essential in scavenging deleterious UV-C irradiation effects, but on the other hand, it acts as a damaging species to biological tissues (Kohen, 1999). Ozone is a non-radical specie, characterized by its sharp odor, that can damage lungs and behave as a powerful oxidizing agent that can directly oxidize biological components

(Halliwell and Cross, 1994; Bocci, 2006). Exposure to ionizing and non-ionizing radiation constitutes another major exogenous source of ROS (Miura, 2004; Okunieff *et al.*, 2008). Exposure of the cell to γ-irradiation results in the production of a whole range of radical and non-radical species from ionization of intracellular water (for example, $OH^{\bullet}$, H_2O_2). Even exposure to non-ionizing radiation such as UV-C (< 290 nm), UV-B (290–320 nm), and UV-A (320–400 nm) can indirectly produce a variety of ROS including $^1O_2$, H_2O_2, and $O_2^{\bullet-}$ radicals, with further production of $OH^{\bullet}$ radicals by hemolytic cleavage of H_2O_2 caused by the UV radiation (Pentland, 1994). Air pollutants such as car exhaust, cigarette smoke, and industrial contaminants encompassing many types of NO derivatives constitute major sources of ROS that attack and damage the organism either by direct interaction with skin or following inhalation into the lung (Koren, 1995). Drugs are also a major source of ROS (Ray *et al.*, 2001). These drugs include antibiotics that depend on quinoid groups or bound metals for activity (nitrofurantoin), antineoplastic agents as bleomycin, anthracyclines (adreamicine) whose mechanism of activity is mediated via ROS production (Fisher and Aristoff, 1988) and methotrexate, which possess pro-oxidant activity (Gressier *et al.*, 1994). A large variety of xenobiotics, for example toxins, pesticides, herbicides, and chemicals (such as mustard gas, alcohol) produces ROS as a by-product of their metabolism in vivo (Han *et al.*, 2004; Das and Vasudevan, 2007; Mena *et al.*, 2009). The invasion of pathogens, bacteria, and viruses might result in the production of many ROS species by direct release from the invaders or an endogenous response induced by phagocytes and neutrophils (Williams and Kwon, 2004; Fialkow *et al.*, 2007).

Although the exposure ROS is extremely high from exogenous sources, the exposure to endogenous sources is much more important and extensive, because it represents a continuous process during the life span of every cell in an organism. The mitochondrion serves as the major organelle responsible for ROS production and many events throughout the cell cycle (Richter *et al.*, 1995; Melov, 2000; Kowaltowski *et al.*, 2009; Izyumov *et al.*, 2010). The reduction of oxygen to water in the mitochondria for ATP production occurs through the donation of 4 electrons to oxygen to produce water (Fleury *et al.*, 2002). During this process, several major oxygen derivatives are formed (Miwa and Brand, 2003; Andreyev *et al.*, 2005) with the possibility of leakage of these ROS from

the mitochondria into the intracellular environment (Fleury *et al.*, 2002). The massive production of mitochondrial ROS is increased further with aging, where the mitochondrion function is impaired and its membrane integrity damaged (Friguet *et al.*, 2008; Ma *et al.*, 2009). Enzymes comprise another endogenous source of ROS. While most enzymes produce ROS as a by-product of their activity, exemplified by the formation of superoxide radicals by xanthine oxidase (Canas, 1999), there are some enzymes designed to produce ROS, such as nitric oxide synthase that yields $NO^{\bullet}$ radicals, those that produce H_2O_2, and those responsible for hydroxylation (Hanukoglu, 2006; Groeger *et al.*, 2009; Leto *et al.*, 2009). White blood cells, including neutrophils, eosinophils, basophils, and mononuclear cells (monocytes), and lymphocytes, with their mechanisms to combat bacteria and other invaders (Fialkow *et al.*, 2007; Larbi *et al.*, 2007), are major producers of endogenous ROS and other factors that act synergistically with ROS (Ginsburg, 1998). Following stimulation, these cells undergo a respiratory burst characterized by a 20-fold increase in oxygen consumption, which is accompanied by an increase in glucose utilization and production of reduced NADPH by the pentose phosphate pathway (Babior *et al.*, 2002). NADPH serves as a donor of electrons to an activated enzymatic complex in the plasma membrane and this NADPH oxidase complex utilizes electrons to produce superoxide radicals from the oxygen molecule. Following dismutation, the production of H_2O_2 can lead to the metal-mediated formation of $OH^{\bullet}$. Moreover, the presence of the enzyme myeloperoxidase can direct the production of HClO by the interaction between hydrogen peroxides and chlorides (Hawkins *et al.*, 2001).

Finally, numerous pathologies and disease states serve as sources for the continuous production of ROS (Ames *et al.*, 1993; Gutteridge, 1993; Dhalla *et al.*, 2000; Halliwell, 2001; Giasson *et al.*, 2002; Warnholtz *et al.*, 2004; Kaneto *et al.*, 2006; Giustarini *et al.*, 2009; Kaneto *et al.*, 2010). More than 200 clinical disorders have been described in the literature in which ROS were important for the initiation stage of a disease or produced during its course. ROS may be important initiators and mediators in many types of cancer, heart diseases, endothelial dysfunction, atherosclerosis and other cardiovascular disorders, in inflammation and chronic inflammation, burn, intestinal tract diseases, brain degenerative impairments, diabetes, eye diseases, and ischemic and post-

ischemic situations. In normal physiological conditions, ROS are produced and play a role in the progress of the aging process where ROS production significantly increases, as well as in the early stages of embryonic development (Lee and Wei, 2001; Dennery, 2007; Covarrubias *et al.*, 2008).

1.1 Protein oxidative damage

The continuous efflux of ROS from endogenous and exogenous sources can result in continuous and accumulative oxidative damage to cellular components (Stadtman and Berlett, 1997; Humphries *et al.*, 2006; Pitt and Spickett, 2008) and, consequently, can alter many cellular functions (Stadtman, 2004). Among the main biological targets, the most vulnerable to oxidative damage are proteins (Davies, 1987; Dean *et al.*, 1997; Stadtman and Berlett, 1997; Dalle-Donne *et al.*, 2005), lipids (Leeuwenburgh and Heinecke, 2001; Muralikrishna Adibhatla and Hatcher, 2006) and DNA (Hemnani and Parihar, 1998; Bertram and Hass, 2008) (Figure 1.2). Nevertheless, proteins are major targets for ROS-induced oxidative damage, as these are the major component of most biological systems. It has been estimated that proteins are able to scavenge 50 to 75% of reactive species such as $OH^{\bullet}$ (Davies *et al.*, 1999).

Among the various ROS, $OH^{\bullet}$, $RO^{\bullet}$, and nitrogen-reactive radicals predominantly cause protein damage. Hydrogen peroxide itself and superoxide radicals in physiological concentrations exert weak effects on proteins, with the exception of those containing SH groups that can undergo oxidation following interaction with H_2O_2 (Davies, 1987; Stadtman and Levine, 2003).

Exposure of proteins to ROS may alter the primary, secondary, tertiary and/or quaternary protein structure, resulting in major physiological changes. Proteins can undergo direct damage following interaction with ROS or indirect damage by reaction with secondary by-products of oxidative stress. This damage can occur via different mechanisms resulting in peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid (Berlett and Stadtman, 1997; Dean *et al.*, 1997; Stadtman and

Berlett, 1997; Davies, 2005). Most protein damage is non-repairable and oxidative changes in protein structure can have a wide range of downstream functional consequences. These consequences can be inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, interference with the creation of membrane potentials, and altered immunogenicity (Dean *et al.*, 1997; Grune *et al.*, 2003; Requena *et al.*, 2003; Dalle-Donne *et al.*, 2005). However, not all proteins are equally sensitive to oxidative damage, being oxidation susceptibility

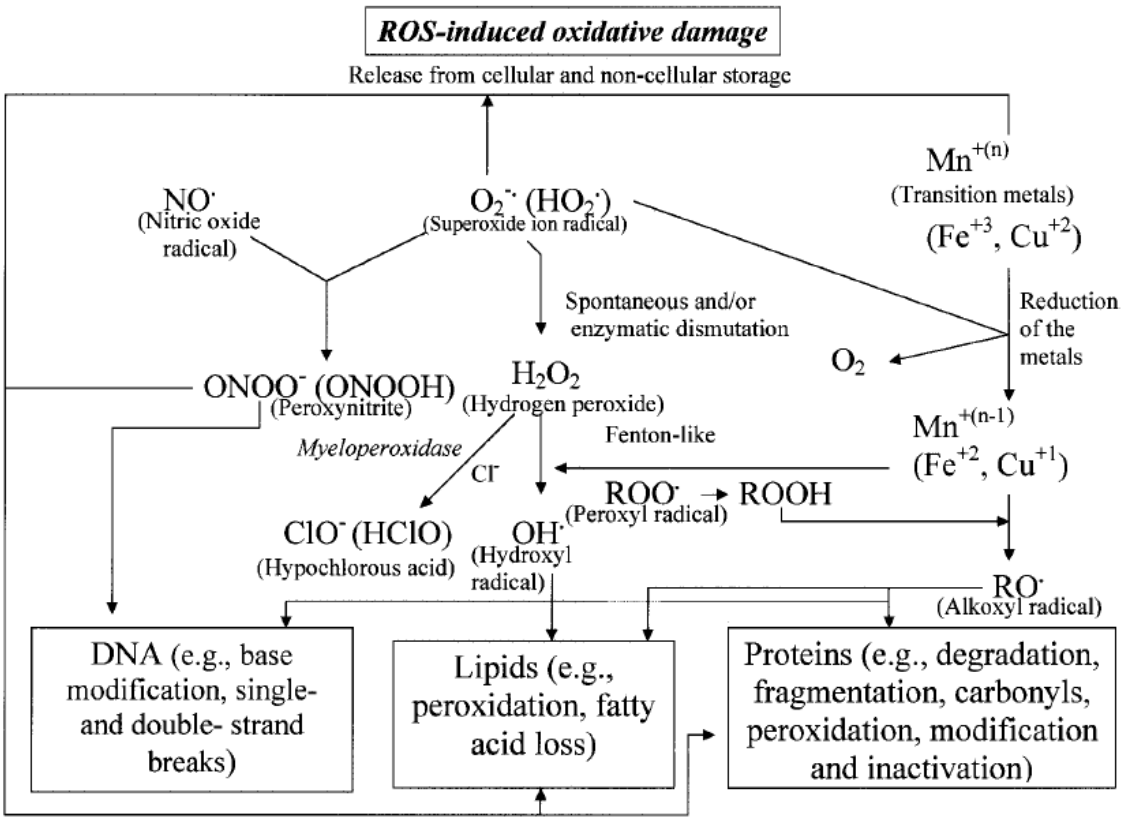

Figure 1.2 – Reactive oxygen species (ROS)- induced oxidative damage to the main biological targets, such as DNA, lipids and proteins (Hrbac and Kohen, 2000).

dependent on protein structure, for example sequence motifs, residues exposed on the molecular surface and bound metal atoms (Stadtman *et al.*, 2003). The importance of oxidized proteins *in vivo* resides not only in its self-consequences,

but also because it can contribute to secondary damage to other biomolecules, for example inactivation of DNA repair enzymes (Halliwell, 2002).

The majority of oxidized proteins are catabolized by proteosomal and lysosomal pathways, but severely damaged proteins appear to be poorly degraded and tend to accumulate in cells (Dean *et al.*, 1997; Grune *et al.*, 2003). The accumulation of such damaged material contributes to an increased oxidative cellular status and to a range of pathological conditions.

1.1.1 Oxidative modifications of proteins by reactive oxygen species

Protein oxidation has been studied and defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress. Until nowadays, reports focusing on the understanding of protein oxidative damage have used agents leading to protein oxidation such as H_2O_2 (Kocha *et al.*, 1997) and HOCl (Yang *et al.*, 1997; Handelman *et al.*, 1998), xenobiotics such as paraquat (Sitte *et al.*, 2000), reduced transition metals such as Fe^{2+} (Berlett and Stadtman, 1997) or Cu^{+} (Allsop *et al.*, 2008), γ-irradiation in the presence of O_2 (Garrison *et al.*, 1962; Davies *et al.*, 1987; Fu and Dean, 1997), UV light (Hu and Tappel, 1992; Voss *et al.*, 2007), ozone (Cross *et al.*, 1992; Berlett *et al.*, 1996), and by-products of lipid and free amino acid oxidation (Requena *et al.*, 1997). Because there are so many mechanisms for induction of protein oxidation and because all of the amino acid side chains can become oxidatively modified, there are numerous different types of protein oxidative modification.

Basic chemical mechanisms involved in free-radical mediated oxidation of proteins were elucidated by pioneering studies of authors such as Swallow (Swallow, 1960), Garrison and co-workers (Garrison *et al.*, 1962), Kopoldova and Liebsier (Kopoldova and Liebster, 1963), and Schuessler and Schilling (Schuessler and Schilling, 1984). In these reports, aqueous solutions of proteins were exposed to ionizing radiation (X rays, gamma rays) under conditions where only $OH^{\bullet}$ and/or $O_2^{\bullet-}$ were formed. They demonstrated these conditions

lead to oxidation of amino acid residue side chains, fragmentation of the polypeptide chain, and formation of protein-protein cross linked aggregates. Although most proteins are not naturally subjected to ionizing radiation, basic principles established in these pioneering studies are applied under more physiological situations where metal-catalyzed reactions mimic the effects of ionizing radiation (Garrison *et al.*, 1970; Butterfield and Stadtman, 1997; Stadtman and Berlett, 1997).

Peptide Bond Cleavage

Hydroxyl radicals formed during exposure to ionizing radiation or by metal-catalyzed cleavage of H_2O_2 are able to abstract the α-hydrogen atom of any amino acid residue of a protein, leading to the formation of a carbon-centered radical (figure 1.3, reaction c). The carbon-centered radical thus formed rapidly reacts with O_2 to form a peroxy radical derivative (figure 1.3, reaction d), which can readily be converted to the peroxide and subsequently to the alkoxyl derivative by reaction with either $HO_2^{\bullet}$ or Fe^{2+} (figure 1.3, reactions e, f, g, h).

The alkyl, alkylperoxyl, and alkoxyl radical intermediates in this pathway may undergo side reactions with other amino acid residues in the same or a different protein molecule to generate a new carbon-centered radical capable of undergoing similar reactions (Berlett and Stadtman, 1997). Moreover, in the absence of oxygen, the carbon-centered radical may react with another carbon-centered radical to form a protein-protein cross-linked derivative (Hawkins and Davies, 2001).

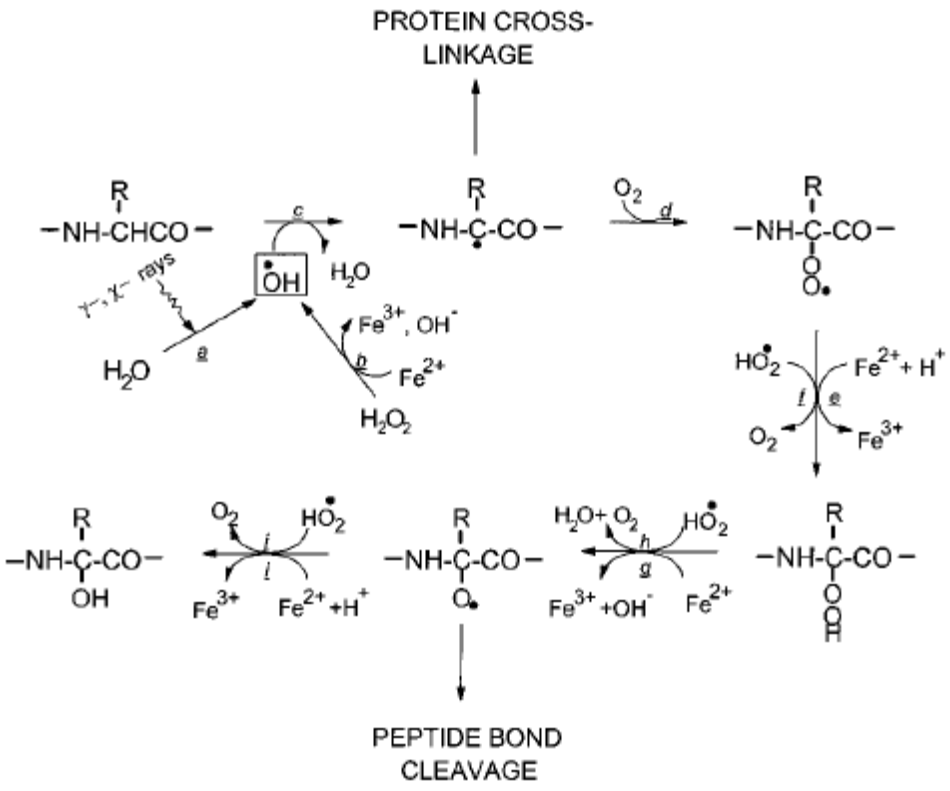

Figure 1.3 – Role of reactive oxygen species in oxidation of the protein backbone (Berlett and Stadtman, 1997).

The generation of alkoxyl derivatives provides the time-point for peptide bond cleavage by either of two different pathways, namely the diamide and the α-amidation pathways (Garrison, 1987). In the diamide pathway (figure 1.4, pathway a), the C-terminal amino acid of the peptide fragment derived from the N-terminal portion of the protein is present as a diamide derivative, whereas the N-terminal amino acid of the fragment derived from the C-terminal portion of the protein is present as an isocyanate structure. In the α-amidation pathway (figure 1.4, pathway b), the C-terminal amino acid residue of the fragment derived from the N-terminal portion of the protein exists as an amide, and the N-terminal amino acid of the fragment derived from the C-terminal portion of the protein exists as an α-ketoacyl derivative. In this case, cleavage by the α-amidation pathway provides a mechanism for the introduction of a carbonyl group into a peptide.

In addition to peptide bond cleavage by these pathways, cleavage can also occur as a consequence of $OH^{\bullet}$-dependent abstraction of a hydrogen atom

from the side chain of glutamyl and aspartyl (Garrison, 1987), as well as prolyl residues (Uchida *et al.*, 1990) of proteins. Based on the observation that the number of peptides formed during radiolysis of proteins is approximately equal to the number of prolyl residues, Schuessler and Schilling (Schuessler and Schilling, 1984) proposed that oxidation of prolyl residues would lead to peptide bond cleavage. This was verified by studies of Uchida and co-workers (Uchida *et al.*, 1990) showing that oxidation of proline residues leads to the formation of 2-pyrrolidone and concomitant peptide bond cleavage. Because acid hydrolysis of 2-pyrrolidone yields 4-aminobutyric acid, the presence of 4-aminobutyric acid in protein hydrolysates represents presumptive evidence for peptide bond cleavage.

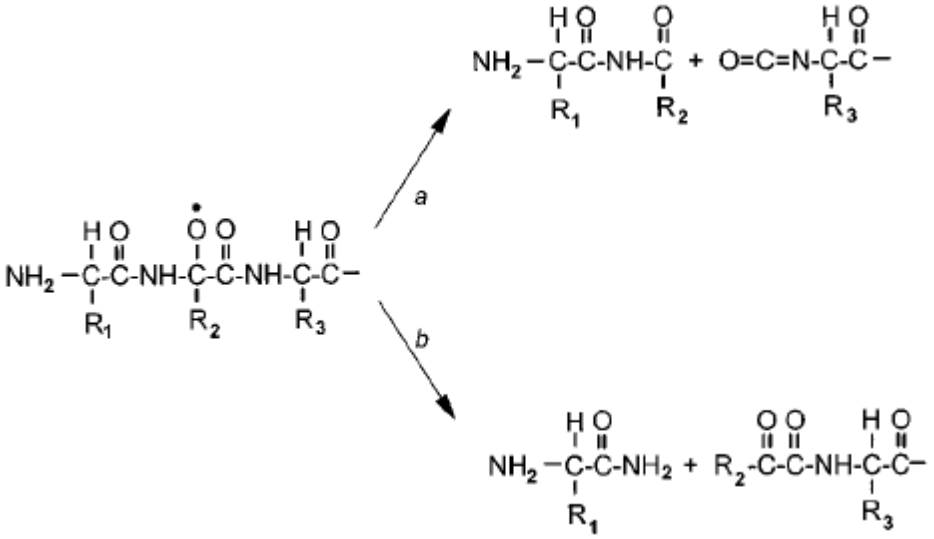

Figure 1.4 – Peptide bond cleavage by the (a) diamide and (b) α-amidation pathways. (Berlett and Stadtman, 1997).

Another kind of peptide bond cleavage comes from the consequence of exposure of proteins to ionizing radiation, which leads to β-scission of amino acid side chains (Dean *et al.*, 1997; Headlam and Davies, 2002). β-scission of residues such as alanine, valine, leucine and aspartic acid leads to the formation of low molecular carbonyl compounds including free formaldehyde, acetone, isobutyraldehyde and glyoxylic acid, respectively. Furthermore, in each case, the

side chain cleavage generates a carbon-centered radical (-NH$^{\bullet}$CHCO-) in the polypeptide chain, as occurs when glycine residues undergo $^{\bullet}$OH-dependent α-hydrogen abstraction.

Oxidation of amino acid residue side chains

The side chains of amino acid residues also represent targets for modification by ROS. Many of these modifications involve a series of reactions analogous to those illustrated in Figure 1.3. Although not fully characterized, the products formed in the oxidation of the most susceptible residues are listed in table 1.2.

The two amino acids that are perhaps the most prone to oxidative attack are cysteine and methionine, both of which contain susceptible sulfur atoms. All oxidizing species can induce modification of cysteine and methionine. Under even mild conditions, cysteine residues are converted to inter- or intra-molecular disulfides, and sulfenic acid, while methionine residues are converted to methionine sulfoxide (MeSOX) residues (Dalle-Donne *et al.*, 2005). Most biological systems contain disulfide reductases and MeSOX reductases that can convert the oxidized forms of cysteine and methionine residues back to their unmodified forms (Berlett and Stadtman, 1997). Considerations of available studies lead to the conclusion that methionine, like cysteine, can function as an antioxidant and serve to protect other functional essential residues of the protein from oxidative damage (Levine *et al.*, 2000). Cysteine residues can be irreversibly oxidized to sulfinic (P-SO_2H) and sulfonic acids (P-SO_3H) by strong oxidative insults, which cannot usually be reversed by metabolic processes and can cause loss of protein function.

Aromatic amino acid residues of proteins also represent prime targets for oxidation by various forms of ROS (reviewed in Dean *et al.*, 1997; Davies *et al.*, 1999). As illustrated in table 1.2, oxidation of phenylalanine residues yields 2-, 3-, and 4-hydroxy derivatives and the 2,3-dihydroxy derivative. Hydroxy radical-mediated oxidation of tyrosine residues gives rise to 3,4-dihydroxy (DOPA)

derivative and also to bi-tyrosine cross-linked derivatives. Oxidation of tryptophan residues leads to N-formyl-kynurenine, 3-hydroxy-kynurenine, kynurenine, and 2-, 4-, 5-, 6-, or 7-hydroxy-tryptophan. Chelating amino acids such as histidine are also susceptible to oxidative attack due to their proximity to forming radicals (Stadtman and Oliver, 1991). Upon oxidation, histidine residues are converted to 2-oxo-histidine, 4-hydroxy glutamate, asparagine and aspartate. With respect to proline residues, these are oxidatively modified to glutamic semialdehyde, pyroglutamic acid, 2-pyrrolidone and 4- and 5-hydroxy derivatives.

Amino acid	Products of oxidation	References
Arginine	Gutamic semialdehyde	Amici *et al.*, 1989
Cysteine	CyS-SCy; CySOH; CySOOH; $CySO_2H$	Garrison, 1987; Brodie and Reed, 1990
Glutamic acid	Oxalic acid; pyruvate adducts	Garrison, 1987
Histidine	2-oxo-histidine; 4-hydroxy glutamate; asparagines; aspartate	Kopoldova and Liebster, 1963; Garrison, 1987
Leucine	3-, 4-, and 5-hydroxyleucine	Garrison, 1987; Fu and Dean, 1997
Lysine	α-aminoadipic semialdehyde; α-aminoadipic acid; N^{ε}-(carboxymethyl)lysine	Amici *et al.*, 1989; Reddy *et al.*, 1995; Daneshvar *et al.*, 1997; Requena *et al.*, 2001
Methionine	Methionine sulfoxide; methionine sulfone	Vogt, 1995; Berlett *et al.*, 1996
Phenylalanine	2-, 3-, and 4-hydroxyphenylalanine; 2,3-hydroxyphenylalanine	Solar, 1985; Davies *et al.*, 1987; Gieseg *et al.*, 1993
Proline	Glutamylsemialdehyde; 2-pyrrolidone; 4- and 5-hydroxyproline; pyroglutamic acid	Amici *et al.*, 1989; Uchida *et al.*, 1990; Kato *et al.*, 1992
Tryptophan	N-formyl-kynurenine;, 3-hydroxy-kynurenine; kynurenine; 2-, 4-, 5-, 6-, and 7-hydroxy-tryptophan	Guptasarma *et al.*, 1992; Pryor and Uppu, 1993; Kikugawa *et al.*, 1994
Tyrosine	3,4-dihydroxyphenylalanine; tyr-tyr cross links	Dean *et al.*, 1993; Giulivi and Davies, 1993; Huggins *et al.*, 1993

Table 1.2 – Oxidation of amino acid residue side chains (Stadtman and Berlett, 1997).

Whereas all amino acid residues of proteins can be naturally subjected to oxidation by ROS, radiation-induced and metal ion-catalyzed oxidation of some amino acid residues lead to the formation of protein carbonyl derivatives (Levine *et al.*, 1994). Protein carbonylation is an irreversible oxidative modification and carbonylated proteins are not repaired, being either removed by proteolytic degradation or accumulate as damaged or unfolded proteins (Stadtman and Berlett, 1998). Carbonyl groups (aldehydes and ketones) may be introduced in the protein at different sites and by different mechanisms (Stadtman and Berlett, 1998). Carbonyl moieties are produced on protein side chains, especially of proline, arginine, lysine, and threonine residues (Berlett and Stadtman, 1997). Metal-catalyzed oxidation of proline and arginine residues leads to the formation of glutamic semialdehyde derivatives and oxidation of lysine residues originates the formation of adipic semialdehyde derivatives (Amici *et al.*, 1989; Requena *et al.*, 2001). With respect to threonine residues, these are oxidized to 2-amino-3-keto-butyric acid derivatives (Shi *et al.*, 2007). In parallel, protein carbonyl groups can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, which directs the formation of a peptide with the N-terminal amino acid blocked by an α-ketoacyl derivative (Berlett and Stadtman, 1997). Protein carbonylation can also occur by Michael-addition of reactive aldehydes, generated for example during lipid peroxidation, with the nucleophilic side-chain of cysteine, histidine, or lysine residues, determining the incorporation of an aldehyde/carbonyl group into the peptide chain (Stadtman and Berlett, 1997). Reactive carbonyl groups such as ketoamines, ketoaldehydes, and deoxyosones can also be generated by secondary reaction of the primary amino group of lysine residues with reducing sugars or their oxidation products (glycation/glycoxidation reactions) (Stadtman and Berlett, 1998).

Protein-protein cross linkage

Oxidative modification of proteins can also promote the formation of intra- or inter-protein cross-linked derivatives by several different mechanisms. These mechanisms are illustrated in figure 1.5 and include: (a) direct interaction of two carbon-centered radicals (Garrison, 1987); (b) interaction of two tyrosine radicals (Giulivi and Davies, 1993); (c) oxidation of cysteine sulfhydryl groups (Garrison, 1987); (d) interactions of carbonyl groups of oxidized proteins with primary amino groups of lysine residues in the same or different protein; (e) reactions of both aldehyde groups of malondialdehyde with two different lysine residues in the same or two different protein molecules; (f) interactions of glycation/glycoxidation derived protein carbonyls with either a lysine or an arginine residue of the same or a different protein molecule (Grandhee and Monnier, 1991); (g) interaction of a primary amino group of a lysine residue with protein aldehydes obtained via Michael-addition reactions with lipid peroxidation products (4-hydroxy-2-nonenal) (Uchida and Stadtman, 1993).

Sensitive analytical methods are available for the detection of Tyr-Tyr cross-linkages in proteins, as this kind of cross-link is very commonly observed as a product of protein oxidation (Heinecke *et al.*, 1993; Kato *et al.*, 2001). However, as shown in figure 1.5, carbon-carbon cross-links can involve interactions of carbon-centered radicals of any two identical or non-identical amino acid residues in the same or different protein molecules. The relevance of this line of research relies on the fact the formation of cross-linked complexes have important implications in the accumulation of oxidized proteins, which occurs in aging and pathological conditions, because cross-linked proteins are often resistant to proteolytic degradation (Grune *et al.*, 1995).

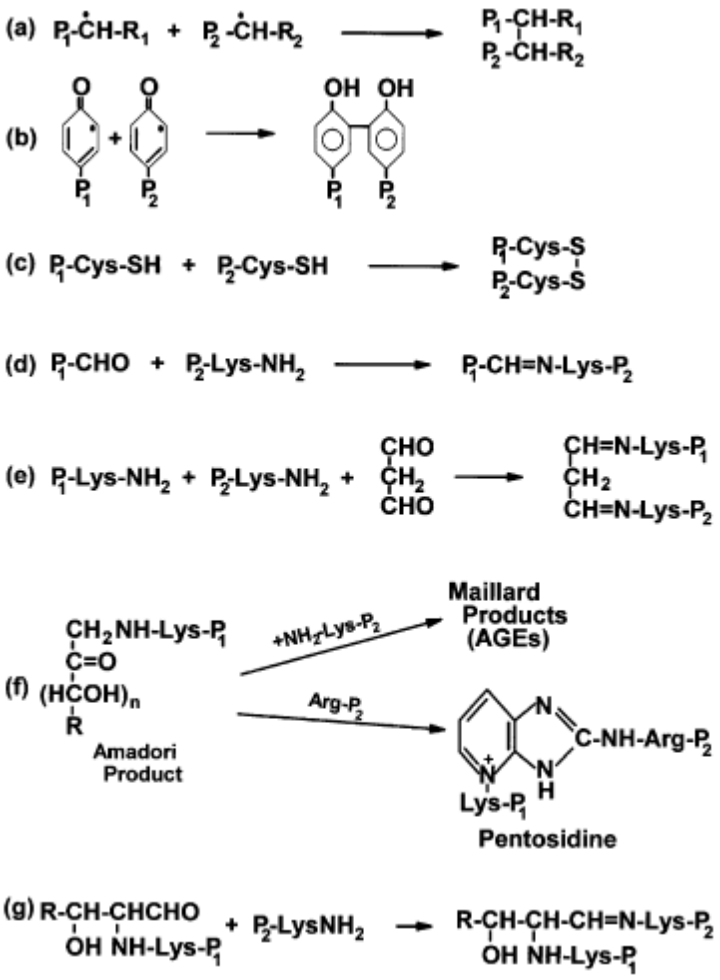

Figure 1.5 – Formation of protein–protein cross-linked derivatives in result of protein oxidative damage (Stadtman and Levine, 2003).

1.1.2 Biological relevance of protein oxidative modifications

Because proteins have many different and unique biological functions, oxidative modifications to proteins can lead to diverse functional consequences. While some biochemical pathways require free radical participation and transient oxidative modification of cellular components, in aging and pathological conditions, oxidative stress leads to a more long-lasting oxidative modification of proteins. In general, with oxidation there is a diminution, if not loss, of protein function (Butterfield *et al.*, 2003).

Oxidative modification of enzymes can consequently cause inhibition of a wide array of enzyme activities (Stadtman, 1990). Enzymes that are particularly sensitive to inhibition by metal-catalyzed oxidation generally have a metal in or near the active site (Stadtman, 1990; Dean *et al.*, 1997). Oxidative modification of enzymes can have either mild or severe effects on cellular or systemic metabolism, depending on the percentage of molecules that are modified and the chronicity of the modification. Several groups have demonstrated that certain enzymes become oxidatively modified during aging (Oliver *et al.*, 1987), including glutamine synthetase (Daggett, 1987), mitochondrial aconitase (Yan *et al.*, 1997), adenine nucleotide translocase (Yan and Sohal, 1998), and carbonic anhydrase (Cabiscol and Levine, 1995). The first three showed an increase in protein carbonyls, whereas the last showed an increase in glutathiolation. Adenine nucleotide translocase and aconitase are both critical enzymes for normal mitochondrial function.

Modification of structural proteins can also lead to a loss of function. For example, when the plasma protein fibrinogen is oxidized either by treatment with an iron/ascorbate radical-generating system or by ionizing radiation, it loses the ability to form a solid clot (Shacter *et al.*, 1995). Another example is α-1-antitrypsin, a plasma protein with primary responsibility in inhibiting proteolysis in tissues such as lung and cartilage. Oxidative modification of a critical methionine residue in α-1-antitrypsin causes loss of function, which seems to contribute to tissue destruction seen in emphysema (Li *et al.*, 2009). The primary oxidant responsible for this modification can be HOCl produced by inflammatory neutrophils (Summers *et al.*, 2008). Plasma low-density lipoprotein (LDL) has been demonstrated to undergo several different types of oxidative modification (Requena *et al.*, 1997; Van Campenhout *et al.*, 2005), leading to the formation of carbonyl groups, aggregation, and increased cellular uptake by tissue macrophages through the scavenger receptor (Hazell and Stocker, 1993; Hazell *et al.*, 1994). Importantly, oxidatively modified LDL has been found in atherosclerotic tissues, suggesting that oxidation of LDL may play a significant role in the etiology of atherosclerosis (Jenkins *et al.*, 2004). Similarly, oxidation of crystalline proteins in the lens of the eye probably plays a role in cataractogenesis (Fu *et al.*, 1998; Molnar *et al.*, 2005).

Generalized increases in oxidized proteins have been found in other disease states, but in many cases the specific proteins have not been identified. There is also the question as to whether there are repair mechanisms for oxidized proteins, mechanism analogous to those that exist for reversal of oxidative DNA damage. The only true enzymatic repair mechanisms that have been identified are restoration of methione residues from methionine sulfoxide catalyzed by methionine sulfoxide reductase and restoration of cysteine sulfhydryls from disulfides accomplished by cellular reducing equivalents such as glutathione (Dalle-Donne *et al.*, 2005). At present, there are no other known mechanisms for reverting oxidative damage to proteins. Damaged proteins are removed from the cell or tissue by proteolysis. Specific removal of oxidized proteins is primarily carried out primarily by the proteasome, which distinguishes between protein oxidized and native forms (Grune *et al.*, 1997; Bader and Grune, 2006). It has been shown an age-dependent decline in both the proteasome and the lysosomal proteolytic systems (Vittorini *et al.*, 1999; Davies and Shringarpure, 2006), which contributes to the accumulation of damaged proteins. It may be worth noting that some oxidative protein changes will go unnoticed by any repair system. This may occur when one amino acid is converted to another, for example, in the metal-catalyzed conversion of proline to hydroxyproline or glutamate and the conversion of histidine to asparagine and aspartate. Unless these changes result in a recognizable conformational change, they will likely go unnoticed by the proteolytic repair systems. Results from recent studies have shown that whereas mild oxidation of some proteins facilitates their proteolytic degradation, prolonged oxidation converts them into forms not only resistant to degradation, but also able to inhibit the ability of the proteasome to degrade other damaged proteins. It is therefore evident that inhibition of cellular protease activities by overoxidation of proteins has an important role in the accumulation of oxidatively damaged proteins occurring during normal senescence and disease states (Davies and Shringarpure, 2006).

1.1.3 Methods of detection of protein oxidative modifications

Under physiological conditions, ROS are generated at a low amount within cells (Mikkelsen and Wardman, 2003). As already outlined, interference with electron transport or redox environmental challenges may dramatically increase their concentrations, contributing to generate additional reactive species by various metabolic pathways. About 10 different ROS have been identified within the cell: $O_2^{\bullet -}$, $OH^{\bullet}$, $RO^{\bullet}$, $RO_2^{\bullet}$, $HO_2^{\bullet}$, H_2O_2, HOCl, HOBr, O_3, and $^1O_2$ (Dalle-Donne *et al.*, 2005). Direct measurements of species such as $O_2^{\bullet -}$ and H_2O_2 have been reviewed (Tarpey and Fridovich, 2001; Kohen and Nyska, 2002). The most used technique for direct detection of radicals is electron spin resonance (Davies and Hawkins, 2004), which allows the detection of relatively stable radicals. Another technique is the spin trapping method in which a highly reactive radical, such as $OH^{\bullet}$, reacts with a trap molecule to produce a stable radical product that can be evaluated (Mason, 2004; Villamena and Zweier, 2004). However, direct determination of ROS is difficult because these species are generally too reactive and have brief half-lives to allow for a direct determination in biological samples. Since molecular products from oxidative stress are generally more stable than the reactive species that caused their modification, ROS measurements often rely in determining the levels of their oxidation targets, namely DNA, lipids and proteins. Of these, as already outlined, there is no doubt that proteins are major targets for *in vivo* oxidative damage occurring either in intra- or extra-cellular environments. Furthermore, proteins have the potential to show the extent of the oxidative injury and the nature of the oxidation itself.

In the early nineties, protein oxidation was monitored basically through fluorescence alterations (for example, tryptophan), free amino groups and carbonyl groups measurements, gel electrophoresis, and evaluation of alterations in protein solubility, surface hydrophobicity, heat stability and enzymatic activity (Davies and Delsignore, 1987; Davies *et al.*, 1987; Meucci *et al.*, 1991; Ogino and Okada, 1995). In the past few years, protein oxidation has been detected and monitored experimentally through a wide array of tests such as amino acid analysis (Levine *et al.*, 1996), capillary electrophoresis (Dolnik and

Hutterer, 2001), HPLC/UV or mass spectrometric (MS) analysis (Sharp *et al.*, 2003; Bjellaas *et al.*, 2004; Bridgewater *et al.*, 2006; Inoue *et al.*, 2006; Kowalik-Jankowska *et al.*, 2006; Schoneich and Sharov, 2006), immunoassays (Dukan *et al.*, 2000; Ballesteros *et al.*, 2001) and fluorescence assays (Finley *et al.*, 1998). Table 1.3 illustrates some oxidative modifications used as markers of oxidative stress in biological samples and the instrumental approaches taken in the study of these modifications.

Modification	Methods of detection
Disulfides	SDS–gel electrophoresis (±β-ME); DTNB
Thiyl radicals	Electron spin resonance spectroscopy
Glutathiolation	RP-HPLC/mass spectroscopy; IEF; ^{35}S–Cys/Chx/SDS-PAGE
Methionine sulfoxide	CNBr cleavage/amino acid analysis
Carbonyls	DNPH-coupled assays: Western blot/ELISA/immunocyto-chemistry/HPLC/A_{370} Reduction with NaB^3H_3
2-Oxo-His	Amino acid analysis
Dityrosine	Fluorescence; proteolysis or hydrolysis/HPLC
Chlorotyrosine	Hydrolysis/nitroso-napthol/HPLC; HBr hydrolysis-GC/MS
Nitrotyrosine	Immunoassay; hydrolysis/HLPC; HPLC/electrochemical detection
Tryptophanyl	Fluorescence; amino acid analysis (alk. hydrolysis); proteolysis/ MS
Hydroperoxides	KI/I_3^-/spectroscopy; $NaBH_4$/ hydrolysis/OPA–HPLC
Lipid peroxidation adducts	$NaBH_4$/hydrolysis/OPA–HPLC; hydrolysis–GC/MS; DNPH; immunoassays
Amino acid oxidation adducts	$NaCNBH_3$ reduction/hydrolysis/ H^1–NMR/MS
Glycoxidation adducts	Derivitization—GC/MS
Cross-links, aggregates, fragments	SDS–gel electrophoresis; HPLC

Table 1.3 – Methods for detecting oxidative protein modifications (Tetik *et al.*, 2007).

Total amino acid analysis can be useful in yielding data on the nature of oxidation reactions occurring by quantifying the loss of specific amino acids (Hawkins and Davies, 2005). This approach can also enable the simultaneous quantification of some oxidation and modification products, for example, methionine sulfoxide and the advanced glycation end product, S-(carboxymethyl)cysteine (Zeng and Davies, 2005). In this method proteins are hydrolyzed to their constituent amino acids, the resulting free amino groups are derivatized with fluorescent tag (for example, o-phthaldialdehyde) and separated by HPLC. Quantification is performed by fluorescence, relative to amino acid (or oxidation product) standards. Nevertheless, this method is only suitable for isolated or purified proteins and only allows quantification all acid-stable amino acids (all common amino acids except cysteine, cystine, asparagine, and glutamine). Some amino acid residues can be quantified by alternative methods. Trp residues can be determined by direct fluorescence measurements (λex 280 nm, λem 340–345 nm), though it should be noted that the fluorescence of protein Trp residues is sensitive to the local environment, and changes in Trp fluorescence are routinely used to assess protein unfolding (Knott *et al.*, 2003). Other amino acid residues can be quantified by reaction with specific reagents that generate strongly fluorescent derivatives. Fluorescamine and 9,10-phenanthrenequinone (PTQ) can be used to quantify Lys (Bohlen *et al.*, 1973) and Arg (Smith and MacQuarrie, 1978) residues, respectively. These methods have been used to assess the role of Lys and Arg residues in protein glycation and glycoxidation reactions (Knott *et al.*, 2003).

Protein-bound thiols can be quantified by numerous methods; these include spectrophotometric with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Hu, 1994), fluorometric with ThioGlo 1 (Ercal *et al.*, 2001), or MS (Schoneich and Sharov, 2006). These approaches have been extended to studies on complex samples where the proteins are separated using 1D or 2D gels (Baty *et al.*, 2002) or HPLC (Schoneich and Sharov, 2006; Sharov *et al.*, 2006).

Quantification of protein cystine residues can be achieved by careful protein digestion using chemical and enzymatic methods (for example, cyanogen bromide or trypsin), cleaving the protein between half-cystinyl residues under conditions that minimize disulfide bond reduction and exchange. These residues have been routinely studied using MS, particularly fast

atom bombardment (FAB) techniques, after HPLC separation of the peptides resulting from partial digestion (Zhou and Smith, 1990).

Protein modification caused by oxidative damage can also be examined by SDS-PAGE or HPLC due to alterations in protein bands or peaks. However, this approach is usually non-quantitative and of poor sensitivity, taking into consideration that extensive modification is typically required to detect changes. This is particularly the case in biological systems where there are very large numbers of components which may all be damaged to only a limited extent, especially with oxidants with limited selectivity (for example, highly reactive radicals). However, this approach has been employed successfully with oxidants that are highly selective and induce damage only to particular residues. An example is the detection of specific changes on apolipoproteins AI and AII (Pankhurst *et al.*, 2003). Mild oxidation of these proteins in high-density lipoproteins (HDL) or plasma resulted in loss of the native isoforms, and the formation of new oxidized species, assessed by HPLC. Another study reported the formation of albumin disulfide dimmers observed in normal plasma following treatment with various peroxides, as well as, in untreated plasma from patients on hemodialysis using SDS–PAGE and Western blot analysis (Ogasawara *et al.*, 2006). This study suggested the albumin dimers are oxidative products derived from peroxides, and their presence in plasma could represent a marker of oxidative stress.

With respect to detection and quantification of specific oxidation products, modification of aromatic side chains can be used as a sensitive marker of protein oxidation, since they are readily oxidized and often yield stable products that can be readily quantified. As already pointed, Tyr oxidation can yield 3,4-dihydroxyphenylalanine (DOPA), 3-nitrotyrosine, 3-chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine, 3,5-dibromotyrosine, and dityrosine. Similarly, oxidation of Phe yields 2-hydroxytyrosine and 3-hydroxytyrosine. These products can be formed by multiple mechanisms, including $OH^{\bullet}$ and peroxyl radical chemistry, peroxidase-mediated processes, and reactions mediated by HOCl and HOBr (Davies *et al.*, 1999). The majority of these materials are stable products and have been employed for instance as markers of the involvement of myeloperoxidase/eosinophil peroxidase in disease (Winterbourn and Kettle, 2000; Davies *et al.*, 2008). In order to quantify these products three major

techniques have been employed: GC/MS, HPLC coupled to various detectors, and immunological methods (Western blotting/ELISA). Both the GC/MS and the HPLC methods require isolation and hydrolysis of the samples prior to product quantification (Hazen *et al.*, 1997; Leeuwenburgh *et al.*, 1998; Heinecke *et al.*, 1999). GC/MS can provide specific structural information, and has limited problems with co-eluting peaks confounding accurate analysis (Heinecke *et al.*, 1999). Moreover, high sensitivity can be achieved and run times are shorter, allowing rapid analysis of multiple samples. However, sample preparation is more complex than for HPLC approaches, due to the need for multiple derivatization steps to make the materials of interest volatile, and the need for stable, isotopically labeled internal standards (Heinecke, 1999). In the case of HPLC approaches, the less complex sample handling diminishes the risk of artifactual oxidation, and multiple products (together with the parent amino acids) can often be quantified in a single run with appropriate detectors minimizing intrarun variability (for example, DOPA and di-tyrosine). These HPLC methods have been extensively used to quantify protein oxidation products in a wide range of protein samples (Finley *et al.*, 1998; Taylor *et al.*, 2003; Dalle-Donne *et al.*, 2005; Mirzaei and Regnier, 2008).

Another approach for the detection of oxidatively modified proteins is the immunological approach. A number of antibodies, of varying degrees of specificity, have been developed for protein oxidation products, allowing in theory the quantification of oxidant-derived damage by ELISA (Onorato *et al.*, 1998). These methods are semi-quantitative and do not yield absolute values. Different oxidized proteins may react with the antibody in a lesser or greater extent than the standards to which they are compared. Nonetheless, the determination of relative levels of oxidant damage by ELISA can still be a valuable tool. For instance, ELISA methods have been developed to examine 3-nitrotyrosine levels and these have been employed to examine plasma samples from people with various inflammatory conditions compared to healthy controls (Sun *et al.*, 2007), core proteins in proteoglycans in the cerebrospinal fluid of infants with hydrocephalus (Krueger, 2004), and extracellular matrix treated with peroxynitrite/peroxynitrous acid (Kennett and Davies, 2008).

Among the oxidative modifications shown in table 1.3, the most commonly measured products of protein oxidation in biological samples are

protein carbonyls. Protein carbonyls are considered as global markers of oxidative because they can be induced both *in vitro* and *in vivo* by a diverse range of agents, are chemically stable products, and are present and detectable in most biological samples even in physiological conditions. Carbonyls can be formed on most amino acids (Hawkins and Davies, 2001), though some are more prone to carbonyl formation than others; for example, metal-catalyzed protein oxidation results in the majority of carbonyls being formed on Arg, Pro, and Lys residues (Requena *et al.*, 2003). However, it has been shown that different oxidant systems give very different patterns of carbonyl formation, and that both free- and protein-bound carbonyls are generated on oxidized proteins (Headlam and Davies, 2004). Protein-bound carbonyl concentrations can be determined via their reaction with 2,4-dinitrophenylhydrazine (DNPH) to give the corresponding hydrazone, which can be quantified spectrophotometrically at 370 nm (Levine *et al.*, 1994). Protein carbonyl groups derivatized with DNPH can also be detected using antibodies to DNPH in both ELISA and Western blotting experiments (Dalle-Donne *et al.*, 2003). Other methods to quantify include the reduction of carbonyl groups with tritiated borohydride, with the resulting radioactive incorporation quantified by radioactive counting (Lenz *et al.*, 1989; Dalle-Donne *et al.*, 2003).

Off all approaches employed in the study of protein oxidative modifications, the ones involving MS analysis are particularly informative given the wealth of structural information that can be obtained from fragmentation patterns (Fu *et al.*, 1995; Fu and Dean, 1997). Although various strategies can provide quantitative information on the modification extent, only mass spectrometry analysis of modified peptides can be uniquely used for the assignment of the modification to specific amino acid residues (Fonseca *et al.*, 2008; Fonseca *et al.*, 2009). For example, direct LC/MS has been used to detect hydroperoxides formed on amino acids and peptides (Fonseca *et al.*, 2008; Morgan *et al.*, 2008; Ronsein *et al.*, 2008), since these materials are too labile to survive acid, alkaline, or enzymatic digestion, thereby precluding the use of these approaches to detect these materials on intact proteins. Another major advantage of MS approaches is the ability to screen for multiple products (if their masses are known or can be predicted) in single runs. Examples include the determination of the oxidation states of Cys and GSH in biological samples

(Guan *et al.*, 2003), methionine oxidation on intact immunoglobulins (Ren *et al.*, 2008), multiple oxidation markers in LDL isolated from human atherosclerotic plaques (Leeuwenburgh *et al.*, 1997), and oxidized proteins in rat plasma using a combined approach with avidin chromatography and tandem mass spectrometry (Mirzaei *et al.*, 2008a). Furthermore, the high potential of MS approaches is enhanced when combined with previous purification, separation or enrichment of peptide samples. Recently, the use of isotope-coded affinity tagging combined with MS approaches has showed to be a powerful method for determining the sites and extent of Cys oxidation in complex biological mixtures (Sethuraman *et al.*, 2004; Fu *et al.*, 2008).

In the past years the quantitative analytical data specifically on oxidative posttranslational protein modifications *in vivo* are being reported at a relatively slower rate compared with significantly larger numbers of publications reporting on the reactions of reactive oxygen and nitrogen species with proteins *in vitro*. This discrepancy has several reasons. Frequently they represent inherent methodological issues, such as protein low abundance and hydrophobicity (membrane proteins), and chemical instability of protein oxidation products. Moreover, a significant problem for the identification of functionally important protein modifications *in vivo* is protein turnover and protein repair. For example, a specific oxidative modification of a Tyr residue essential for phosphorylation, may be functionally significant; yet, it may never be detected *in vivo* because the biological half-life of the Tyr-modified protein is too short to allow isolation of sufficient material for analysis. Nevertheless, this Tyr-modification could be responsible, for example, for the triggering of a signaling pathway or a specific stress response during its temporary existence. The sensitivity of this particular protein to this Tyr-modification would then be recognized only during an *in vitro* oxidation of the recombinant protein or through the use of cell cultures in which protein turnover is pharmacologically suppressed (Schoneich and Sharov, 2006). Therefore, *in vitro* protein oxidation experiments are of high relevance in the on-going of mechanistic studies and identification of "novel" protein oxidation products.

1.1.4 Protein oxidation as a marker of oxidative stress: advantages and disadvantages

Taking into consideration that proteins are ubiquitous in all cells and tissues, and are susceptible to oxidative modification, they can serve as useful markers of oxidative stress to apply in the study of numerous disease conditions and aging. Compared to measuring products of lipid peroxidation (Montine *et al.*, 2002) and DNA oxidative base modifications (Taghizadeh *et al.*, 2008), proteins offer some advantages as markers of oxidative stress. There are a large number of reported sensitive assays for the determination of oxidatively modified proteins and, therefore from a technical perspective, oxidized proteins serve as a marker for oxidative stress. In general, amino acid oxidation products are more stable when compared to lipid oxidation products during sample storage. For example, protein carbonyls are formed earlier and remain circulating for longer periods in the bloodstream, when compared with other parameters such as MDA (Pantke *et al.*, 1999). Also, their chemical stability is convenient for laboratory measurements and sample storage (Stadtman and Levine, 2003).

Another important feature relies on the fact the nature of the protein modification can give significant information as to the type or source of the oxidant involved in the oxidation process. Such information may help to predict the consequences of oxidation, as well as to provide a basis for designing appropriate interventions to alleviate injury. For example, chlorination of tyrosine residues and amino groups of lysine residues are specific markers of oxidation by HOCl and could reflect neutrophil and/or monocyte involvement in the oxidative stress response (Hazen *et al.*, 1999; Kettle, 1999). This contrasts with lipid peroxidation, where propagation reactions involving the initial lipid oxidation products result in loss of the information on the initial oxidative insult.

The highly specific nature of protein oxidation also leads to one of the disadvantages of using these macromolecules as markers of oxidative stress; that is, there is no single universal marker for protein oxidation. Because so many different protein oxidation products can be formed, it may be necessary to set up several different assays in order to find the most appropriate assay for the

type of oxidative stress involved. For example, singlet oxygen is not a particularly good inducer of protein carbonyls. When this ROS is the source of oxidative stress, the oxidation products to be assayed should be methionine, histidine, tyrosine and tryptofan oxidation products, since these are more susceptible to attack by singlet oxygen (Davies, 2003).

Overall, the selection of a suitable marker of oxidative stress *in vivo* will require some knowledge of the physiological conditions being explored as well as some exercise of trial and error (Launer, 2004).

1.2 Protein glycation and glycoxidative damage

Protein glycation is a slow natural process involving the chemical modification of reactive amino and guanidine functions in amino acids and proteins by sugars and carbohydrates-derived reactive carbonyls. Glycated proteins are formed by non-enzymatic reactions between reducing carbohydrates (for example, glucose, fructose, ribose) or derivatives (ascorbic acid, etc.) with either terminal or amino groups in lysine and arginine residues, with both free α-amino group and free side chains in cysteine and tryptophan residues, and with blocked N-terminus and free side chains in cysteine, lysine, and histidine residues (Munch *et al.*, 1999). This process is distinct from that enzymatically catalysed by glycosyltransferase to synthesize glycoproteins involved in many biological processes (Costello *et al.*, 2007). Glycosylation refers to the attachment of oligosaccharides to specific protein side chains such as asparagine (N-linked), serine and threonine (O-linked), and the C-termini of cell surface proteins.

The mechanism of the glycation reaction was first described by Maillard (Maillard and Gautier, 1912) at the beginning of the 20th century by incubation of amino acids with different sugars. Although it was postulated that the reaction could have important implications in human health, particularly in diabetes, research efforts on the topic in the following years focused primarily

on understanding its chemistry and exploiting the knowledge to improve the sensory attributes of foods, such as color, flavor, taste, and the overall appeal. Moreover, in addition to these beneficial aspects, the Maillard reaction also results in the loss of nutritional quality of food proteins and the formation of deleterious reaction products with genotoxic, mutagenic, and carcinogenic properties (Skog *et al.*, 1998; Persson *et al.*, 2003). It has been shown that these reaction products are orally absorbed through the normal diet and accumulate with the body pool of endogenous formed glycation products, given that they are only partially eliminated in the urine (Koschinsky *et al.*, 1997). The importance of the Maillard reaction does not only apply to foods and food systems. Given the discovery of the evidence the Maillard reaction also takes place *in vivo* (Trivelli *et al.*, 1971), its relevance to human health is of great importance and has gained increasing attention over the past years. *In vivo*, the Maillard reaction is generally called glycation. The glycation reaction between reducing sugars or their derivative and amino-containing biomolecules, such as proteins, DNA and lipids, has been implicated in the development of several age- and diabetes-related pathologies (Monnier *et al.*, 1992; Stitt *et al.*, 2002; Cooper, 2004; Takeuchi and Yamagishi, 2008). With longer exposition to hyperglycemia conditions there is evidence of the role of glycation products in age-related diseases such as atherosclerosis or amyloidosis, and neurodegenerative disorders such as Alzheimer, Parkinson, Pick and Lewy body diseases (Reddy and Beyaz, 2006; Daroux *et al.*, 2010). Nevertheless, much of what is acknowledged to date about the nature of glycation is derived from *in vitro* studies involving amino acids or proteins and simple reducing sugars under high temperature conditions (Cho *et al.*, 2007). In the past years, several *in vitro* studies have been performed under physiological conditions, with the aim of helping to elucidate relevant pathways or mechanisms of the glycation reaction *in vivo*. Changes in structure and function of proteins, such as albumin (Sharma *et al.*, 2002; Ahmed and Thornalley, 2005; Khan *et al.*, 2007), hemoglobin (Selvaraj *et al.*, 2006; Sen *et al.*, 2007), immunoglobulin (Dolhofer-Bliesener and Gerbitz, 1990; Vrdoljak *et al.*, 2004), collagen (Pageon and Asselineau, 2005; Xiao *et al.*, 2007), fibronectin (Tarsio *et al.*, 1985; Cavalot *et al.*, 1996), lens proteins (Swamy *et al.*, 1993; Linetsky *et al.*, 2008), myelin (Vlassara *et al.*, 1984; Ryle and Donaghy, 1995), insulin (Abdel-Wahab *et al.*, 1997; Abdel-

Wahab *et al.*, 1997a), and actin (Syrovy and Hodny, 1993; Kuleva and Kovalenko, 1997) have been ascribed to glycation products formation. Also, there is evidence of metabolic enzymes inactivation as a consequence of protein glycation events (Ulrich and Cerami, 2001). The investigations of protein glycation conducted under physiological conditions have contributed to elucidate the reaction pathways occurring in a similar way *in vivo*, as well as, to identify biomarkers such as Nε-(Carboxymethyl)lysine (CML) and pentosidine that can be used for evaluating the extent of protein glycation in biological systems. Although there is the general consensus that protein glycation in vivo involves a complex cascade of reactions including condensations, rearrangements, fragmentations, and oxidative steps, research conducted so far has enabled the discovery of several types of glycation inhibitors, such as carbonyl trapping compounds, antioxidants and metal ions chelators (Rahbar and Figarola, 2003; Peyroux and Sternberg, 2006). This sets the starting point for the development of new strategies for controlling protein glycation reactions *in vivo* and preventing associated health complications.

1.2.1 Protein glycation

The mechanism of the glycation reaction firstly tested by Maillard at the beginning of the 20th century can be described in three main stages as shown in figure 1.6. In the first one, the reaction between a reducing sugar and an amino group is initiated with the reversible formation of an adduct known as Schiff base. The Schiff base results from the conversion of the aldehydic carbon-oxygen double bond of the sugar to a carbon-nitrogen double bond with the amine. Because the Schiff base is a thermodynamically unstable form in relation to the equilibrium cycled pyranose or furanose forms, it gives rise to an enaminol intermediate by rearrangement and, subsequently, to a relatively stable ketoamine compound known as the Amadori compound (Amadori, 1925). In the second stage, the Amadori compound further undergoes a series of dehydration and fragmentation reactions generating a variety of carbonyl

compounds. These compounds are generally more reactive than the original carbohydrate and act as propagators by reactions with free amino groups.

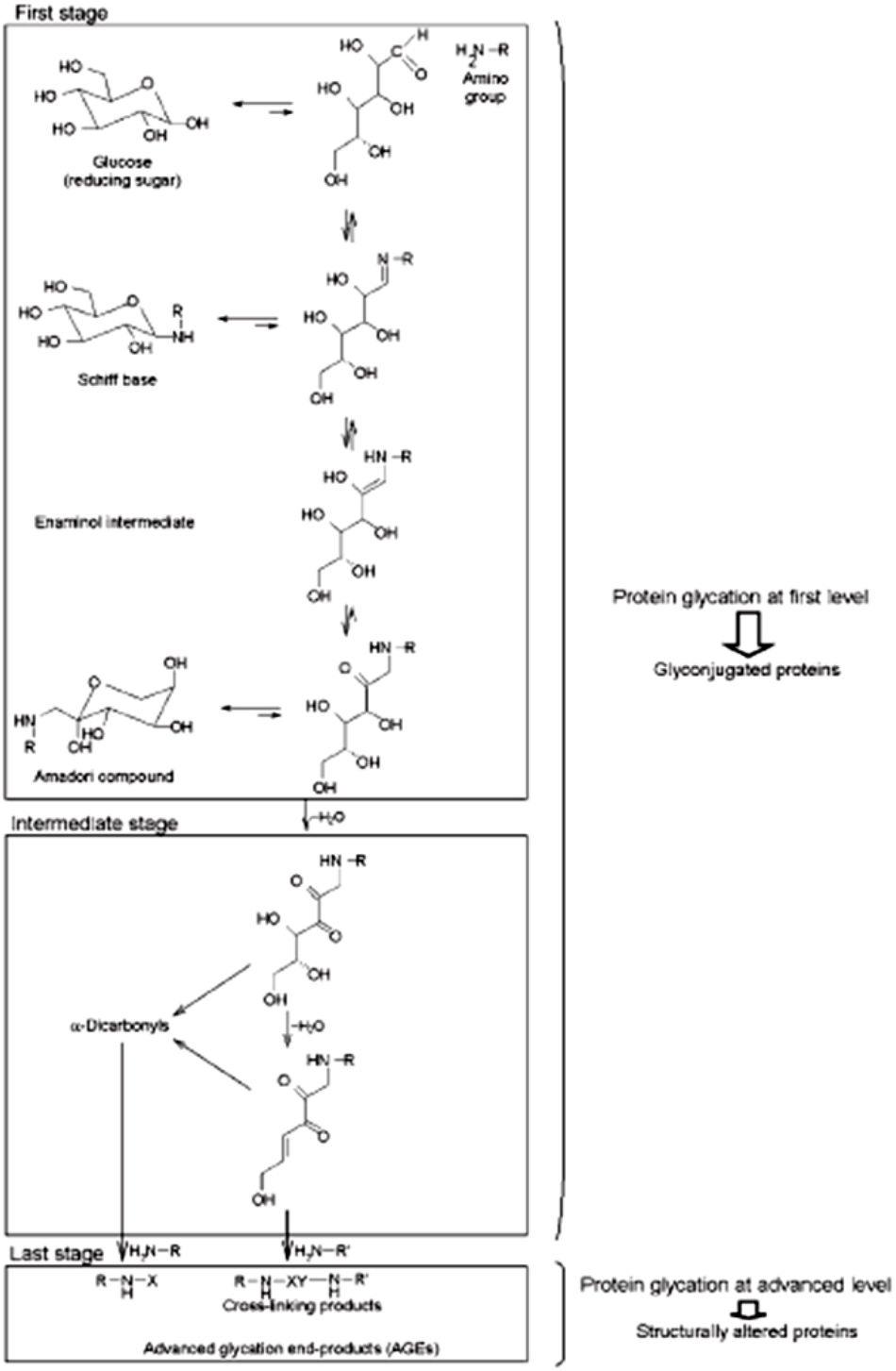

Figure 1.6 – Mechanism of the Maillard reaction with the main stages of the reaction and the significance levels in protein non-enzymatic glycation. R-NH2: protein; X: unknown chemical structures formed by reaction of α-dicarbonyl compounds with other protein or by cross-linking processes.

Among the most active enhancers are α-dicarbonyls such as methylglyoxal, glyoxal, glucosones, deoxyglucosones and dehydroascorbate (Watanabe *et al.*, 2002; Cho *et al.*, 2007). In the final stage, a series of rearrangements lead to the irreversible formation of a variety of heterogeneous structures, commonly known as advanced glycation end-products (AGE), characterized by a wide structural and physicochemical diversity. Till date, a variety of AGEs have been discovered, for example, pentosidine, pyrraline, argpyrimidine, tetrahydropyrimidine, carboxymethyllysine, carboxyethyllysine, a number of imidazolones and lysine-lysine crosslinks (imidazolysine, MOLD, and GOLD) (Ulrich and Cerami, 2001).

The glycation process is of particular interest when the amino group is part of a protein. Structure, biological function and turnover of proteins are altered when protein glycation takes place (Ulrich and Cerami, 2001). The formation of the Amadori product from the Schiff base, which represents the first level of protein glycation, is characterized by its slow kinetic and natural occurrence in the human body (Brock *et al.*, 2003). However, these modifications are accelerated under pathophysiologic conditions being strongly associated with high concentration of glucose or hyperglycemia. For this reason, glycation has often been related to chronic complications of diabetes mellitus, renal failure and degenerative changes occurring in the course of aging (Baynes, 2001; Hipkiss, 2006). The involvement of glycation in mentioned diseases has raised a great medical interest in preventing the glycation of proteins in human. For this purpose, it is very relevant to gain knowledge about the mechanism of the reaction. The initiation of the glycation process depends primarily on the nucleophilicity of the amino group. In the case of proteins, glycation takes place preferentially at a ω-amino group of amino acids and at the α-amino group of the amino-terminal residue of the proteins. In general, amino groups with lower pKa values such as lysine and arginine (10.53 and 12.48, respectively) present higher nucleophilicity and, therefore, should be expected to be more reactive toward glycation (Bunn *et al.*, 1979). However, other factors have to be considered since the site selectivity of glycation has been ascribed to the Amadori rearrangement step (Shilton *et al.*, 1993). It has been suggested for lysines that the properties of nearby amino acids play a role in determining whether a given lysine is glycated or not. It has been reported

that positively charged amino acids located close to a lysine residue (in either the primary or three-dimensional structure) catalyze the glycation of that lysine and the Amadori rearrangement (Iberg and Fluckiger, 1986). On the other hand, the formation of hydrogen bonds between lysine residues and other amino acids could partially protect lysines from being glycated. This selectivity could justify the selective targeting of AGE formation toward proteins (Nacharaju and Acharya, 1992) and proofs of the existence of preferential sites of glycation in a protein have been recurrently reported (Iberg and Fluckiger, 1986; Calvo *et al.*, 1993; Lapolla *et al.*, 2004; Hinton and Ames, 2006).

All reducing carbohydrates can potentially be involved in the Maillard reaction. Pentoses such as arabinose or ribose, as well as, hexoses such as glucose, fructose, or galactose, and even disaccharides as lactose or maltose have been encountered in studies related to protein glycation (Dyer *et al.*, 1991; Fenaille *et al.*, 2004). The physiologically relevant D-glucose is one of the least reactive sugars in protein glycation, which could be a possible reason for its evolutionary selection as the main free sugar in vivo (Bunn *et al.*, 1978). On the other hand, D-ribose is the sugar counterpart in most of the in vitro studies of protein glycation, despite its levels in humans are not elevated. D-ribose is preferred for *in vitro* experiments because it is known to be more reactive in the Maillard reaction than D-glucose, probably due to a higher fraction of the more reactive acyclic form (Yeboah *et al.*, 1999).

As already pointed, in the case of reducing sugars, the intermediate Schiff undergoes rapid cyclization to form the unstable N-substituted glycosylamine, which in turn undergoes Amadori rearrangement to produce the relatively stable 1-amino-1-deoxy-D-fructose. Subsequently, Amadori rearrangement involves the acid-catalyzed ring opening to give the iminium ion which undergoes deprotonation to form the enaminol, and upon tautomerism produces the Amadori product. The Amadori rearrangement can also be catalyzed by transition metal ions, via the same mechanism as protons (Cho *et al.*, 2007). Examples of Lewis acid-catalyzed Amadori rearrangement have been reported (Hayase *et al.*, 1996), in which the transition metal ions are more efficient than protons, since they are usually polyvalent and can form multiple bonds with its substrate. With regards to the cyclic glycosylamine, metal ions such as Fe^{2+}, Fe^{3+}, or Cu^{2+} accelerate the formation of the reactive acyclic

iminium ion, thus increasing the reaction rate of the Amadori rearrangement. So, although Amadori products are fairly stable, with time and under appropriate conditions they can either reverse to the starting substrates or decompose to form AGEs (Zyzak *et al.*, 1995). The degradation of Amadori products may occur both under non-oxidative and oxidative conditions. At pH 7.4 and 37°C, the reversal of Amadori product to the starting substrates accounts for more than 90% of its degradation under non-oxidative conditions (Zyzak *et al.*, 1995). On the other hand, in the pH range of 4-7, the formation of α-dicarbonyl compounds, including 1-deoxyglucose, 3-deoxyglucosone, and 1-amino-1,4-dideoxy-2,3-oxo-glucosone, is a major degradative pathway. Further degradation of 3-deoxyglucosone via a retro-aldol reaction generates methylglyoxal and glyceraldehyde (Thornalley *et al.*, 1999). One of the major consequences *in vivo* of protein-bound dicarbonyl moieties is the formation of inter- and intra-protein cross-links, a process responsible for many age and diabetes related health complications. Among the identified α-dicarbonyl compounds, 3-deoxyglucosone is known to rapidly react with protein amino groups to form AGEs such as Nε-(Carboxymethyl)lysine (A) or representative cross-links, such as imidazolone (B), pyrraline (C), and pentosidine (D) presented in figure 1.7 (Thornalley *et al.*, 1999).

The oxidative cleavage of Amadori product has been proposed as a major pathway for CML *in vivo* formation (Glomb and Monnier, 1995). CML is one of the central AGEs, it is used as a biomarker for oxidative stress and long-term protein damage in normal aging and has been found in proteins and in free form *in vivo* (Ahmed *et al.*, 1997). It may arise either from reaction of lysine residues with glyoxal derivatives (Glomb and Monnier, 1995) or from autoxidation of early-stage AGES such as the Amadori product.

Another pathway of AGE formation is the Namiki pathway which comprises the degradation by retro aldol cleavage reactions of the Schiff base formed during the initial stage of the glycation reaction (Hayashi and Namiki, 1980). It results in the formation of short chain carbonyl compounds such as glyoxal, glycoaldehyde, and their corresponding imine analogs (Glomb and Monnier, 1995). The rate of Schiff base degradation via the Namiki pathway increases with increasing pH, as retro-aldolization is favored under basic conditions. Glomb and co-workers have shown that lysine derivatives of glyoxal

and glycolaldehyde are formed under physiological conditions (Glomb and Monnier, 1995). Glyoxal and its corresponding imine analogs are responsible for the formation of several AGEs including CML, (carboxymethyl)arginine (CMA), N^{ε}-(carboxyethyl)lysine (CEL), glyoxal-lysine dimer (GOLD), arginine-lysine-glyoxal crosslink (GODIC) and pentosidine.

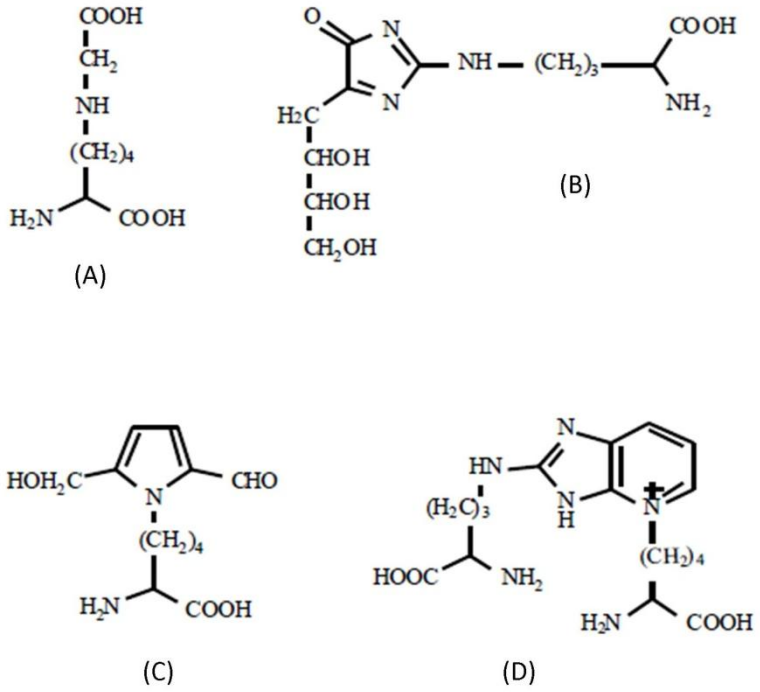

Figure 1.7 – Known structures of AGEs such as CML (A), imidazolone (B), pyrraline (C), and pentosidine (D) (Cho *et al.*, 2007).

However, the generation of α-dicarbonyls involved in the formation of AGEs occurs beyond protein glycation. Simple monosaccharides autoxidize at pH 7.4 and 37°C, resulting in the production of β-ketoaldehydes via a process involving free radicals (Wolff and Dean, 1987). Enolization is a pre-requisite for monosaccharide autoxidation, and trace metal ions may catalyse monosaccharide autoxidation. Spontaneous degradation of glucose in buffered solution at 37°C leads to glyoxal, methylglyoxal and 3-deoxyglucosone (Thornalley *et al.*, 1999).

Despite their relatively low concentration compared to the concentration of the parent carbohydrates, α-dicarbonyls are the class of intermediates

responsible for most of the AGE formation, including a significant number of crosslinks, due to their highest reactivity. Besides the AGEs already shown in figure 1.7, a series of other compounds, mainly cross-links resulting from α-dicarbonyls have been identified and are presented in figures 1.8 and 1.9.

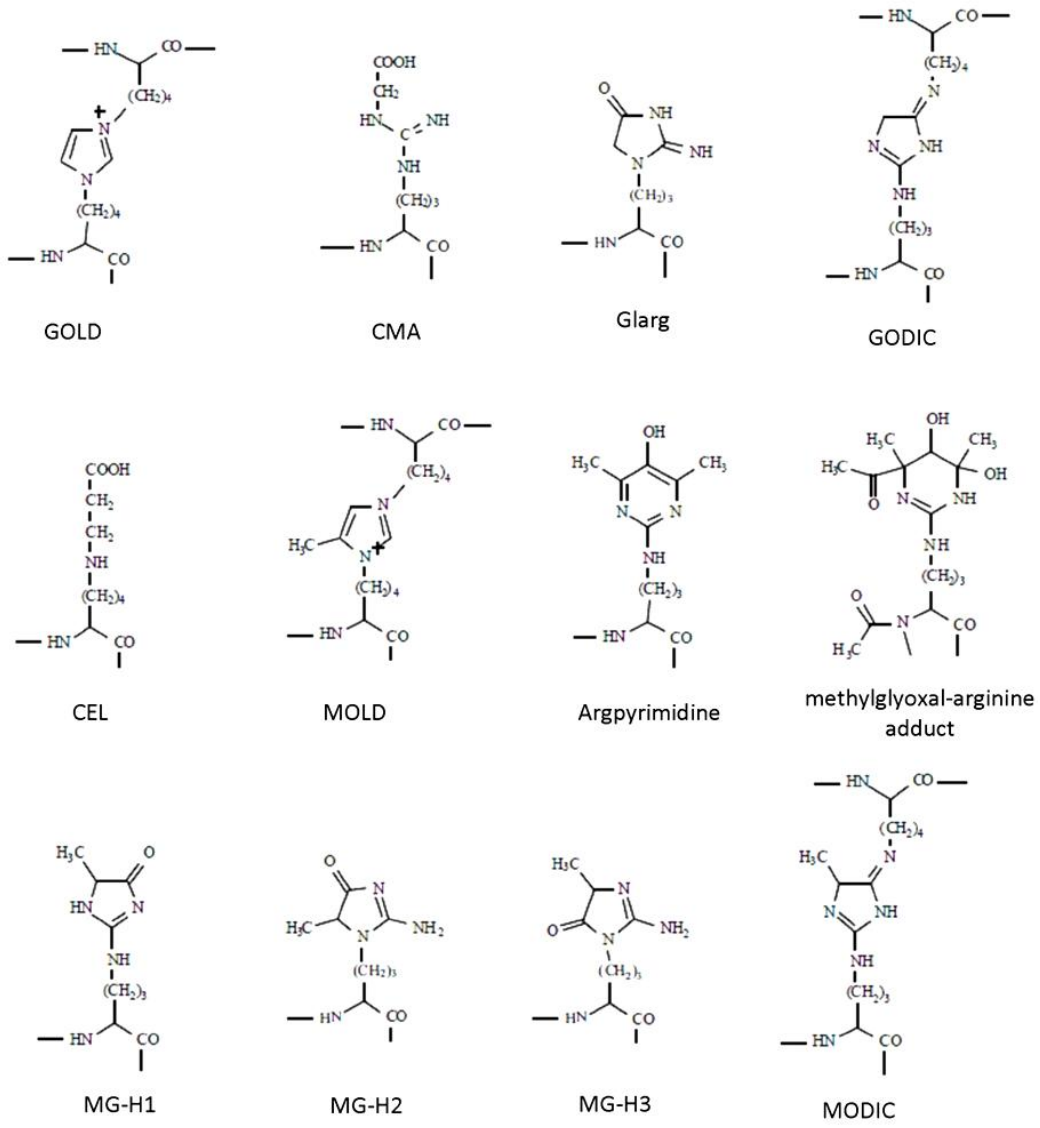

Figure 1.8 – Structures of known AGEs resultant from the propagation of the glycoxidative damage to proteins after the formation of α-dicarbonyls (Cho *et al.*, 2007).

Glyoxal was identified as an intermediate able to react with the ε-amino group of lysine residues in proteins to form CML and an imidazolium crosslink known as GOLD (figure 1.8) (Chellan and Nagaraj, 1999), which has been detected in human serum and lens protein. The modification of the guanidine

function in arginine residues by glyoxal leads to CMA (figure 1.8) (Iijima *et al.*, 2000; Odani *et al.*, 2001), which was found in skin collagen and human serum protein, and to an imidazolone designated as Glarg (figure 1.8) (Paul *et al.*, 1998). GODIC (figure 1.8) was identified as an arginine-lysine-glyoxal crosslink by Lederer and co-workers from a D-glucose-BSA incubation mixture (Lederer and Klaiber, 1999), and was later found *in vivo* (Biemel *et al.*, 2001).

Methylglyoxal irreversibly modifies lysine residues in proteins under physiological conditions to give CEL (figure 1.8) (Ahmed *et al.*, 1997), and an imidazolium crosslink known as MOLD (figure 1.8) which has been found in human lens protein (Frye *et al.*, 1998) and human serum (Odani *et al.*, 1998). Adducts of methylglyoxal with arginine residues have also been reported. Argpyrimidine (figure 1.8) was acknowledged as a major fluorescent AGE in glycated bovine lenses (Shipanova *et al.*, 1997) and detected later in tissue proteins (Wilker *et al.*, 2001), human lens proteins (Kim *et al.*, 2010), and in human cancer tissues (van Heijst *et al.*, 2005). A methylglyoxal-arginine adduct having a tetrahydropyrimidine structure (figure 1.8) was also identified as a non-fluorescent product (Oya *et al.*, 1999), along with methylglyoxal-derived hydroimidazolone MG-H1 (figure 1.8), and the isomeric hydroimidazolones MG-H2 and MG-H3 (figure 1.8) have also been reported (Ahmed *et al.*, 2002). The arginine-lysinemethylglyoxal crosslink named as MODIC (figure 1.8) is an analog of GODIC, was synthesized and detected in a D-glucose-BSA incubation mixture (Lederer and Klaiber, 1999). However, this compound has been detected in human serum albumin and lens proteins (Biemel *et al.*, 2001).

With regards to AGEs comprising 3-deoxyglucosone-modified amino acid residues, Skovsted and co-wokers have isolated a derivative of the imidazolium crosslink 3-deoxyglucosone-lysine DOLD (figure 1.9), a result from the reaction between N2-hippuryl-lysine and 3-deoxyglucosone (Skovsted *et al.*, 1998). Hydroimidazolone (figure 1.9) have also been reported to result from the reaction between 3-deoxyglucosone and arginine derivatives (Jono *et al.*, 2004), being immunohistochemically detected in diabetic patients (Niwa *et al.*, 1997), patients with chronic renal failure and reumatoid arthritis, as well as *in vitro* human serum albumin incubated with glucose (Franke *et al.*, 2000).

Other crosslinks that have been narrowly reported in the literature are the acid-stable cationic fluorophores vesperlysines (figure 1.9). These

compounds were isolated as major fluorescent AGEs after hydrochloric acid hydrolysis of AGE-BSA (Nakamura *et al.*, 1997), and identified as crosslinked products from two lysine side-chains in proteins. They were considered to be generated from lysines and oxidative degradation of glucose, as is the case of glycoxidation products like pentosidine. Besides α-dicarbonyls, hydroxyaldehydes such as glycoladehyde and glyceraldehyde have also been shown to be the starting materials for the recently discovered AGEs GA-pyridine (figure 1.9) (Greven *et al.*, 2005), OP-lysine (figure 1.9) (Argirov *et al.*, 2005) and Glyceraldehyde derived Pyridinium compound (GLAP) (figure 1.9) (Usui and Hayase, 2003).

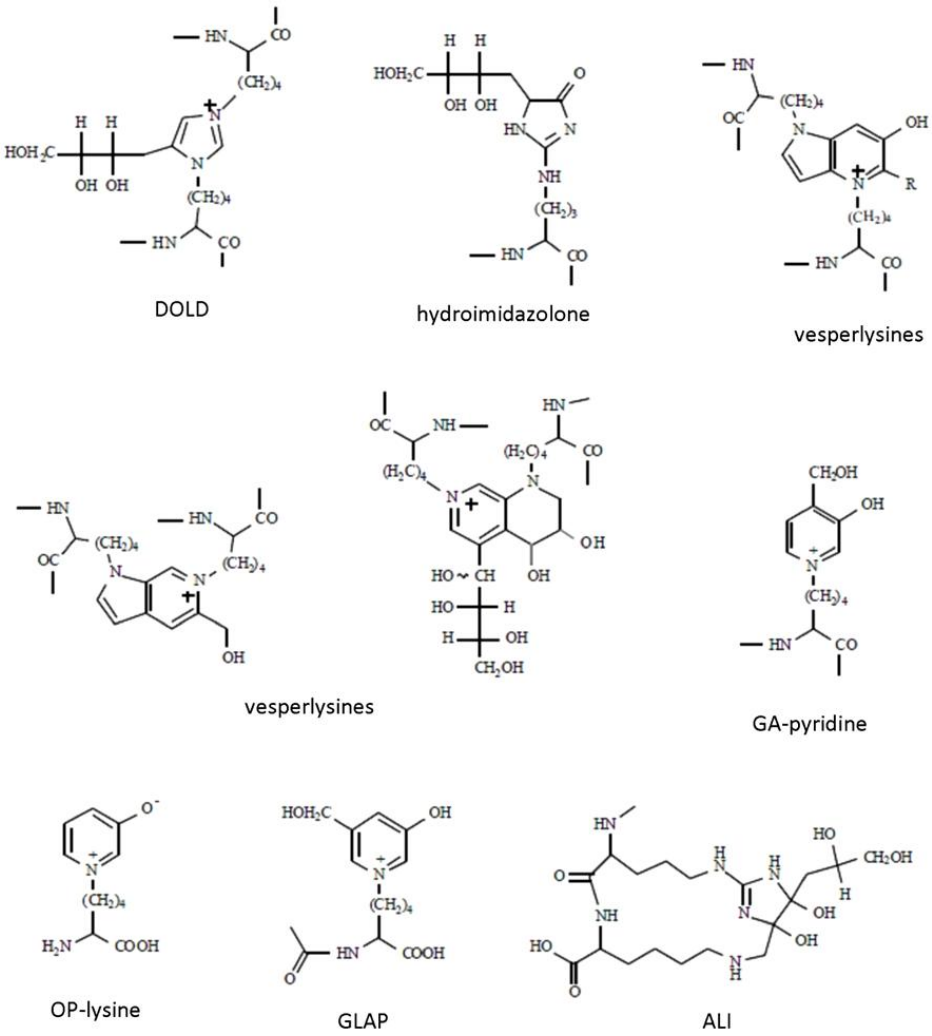

Figure 1.9 – Structures of known AGEs resultant from the propagation of the glycoxidative damage to proteins after the formation of α-dicarbonyls (Cho *et al.*, 2007).

Immunological approaches using monoclonal and polyclonal antibodies specific for AGE-modified proteins have been developed to confirm the presence of AGEs *in vivo*. CML was shown to be a major immunological epitope among AGEs (Ikeda *et al.*, 1996), with various reports in the literature focusing on the preparation of monoclonal anti-AGE antibodies specific for CML due to its structural similarity for instance with CEL. Several polyclonal anti-AGE antibodies specific for other epitopes (non-CML) have also been prepared and characterized (Ikeda *et al.*, 1998). The antibodies developed for glyoxalmodified proteins are able to recognize GOLD; the ones prepared for methylglyoxal-modified proteins identify argpyrimidine, MOLD, and GOLD. Another series of non-CML anti-AGE antibodies that can recognize a broad range of serum proteins modified by 3-DG, glyceraldehyde, glycolaldehyde, methylglyoxal, glyoxal, and glucose has also been developed (Takeuchi *et al.*, 2000).

Another critical property of AGE is their ability to activate receptors for advanced glycation end products (RAGE), a signal transduction receptor of the immunoglobulin family (Li and Schmidt, 1997). Initially, RAGEs were thought to be scavenger receptors implicated in the removal of AGEs, but the characterization of RAGE provided evidence that binding of AGEs to RAGE did not lead to an accelerated clearance and degradation with all the expected beneficial effects. Instead, Ramasamy et al. hypothesized that, due to such interaction, AGE induce a potent impact in tissues stimulating processes linked to inflammation and further consequences (Ramasamy *et al.*, 2005). Therefore, targeting this pathway may represent one of the logical steps in the prevention and treatment of the disorders associated with AGEs formation and accumulation.

1.2.2 Analysis of glycated proteins

The analytical set of equipment, methods, and techniques available for studying non-enzymatically-modified proteins such as glycated proteins involves practically all currently available technologies. First it is possible to study the

altered proteins as such. In this respect, collagen, eye crystallins, plasma and blood proteins, like haemoglobin, represent targets of many studies and are routinely assayed in diabetology. The other possibility is to look for marker compounds (hydrolytically stable) which may serve as indicators of the non-enzymatic protein modifications. Nevertheless, besides the minute quantities of such compounds in biological samples, there is the need to take into account the large number of compounds arising from protein glycation that can be structurally unstable, insoluble or resistant to hydrolysis. On the other hand, some of these compounds can possess a characteristic luminescence, which can help to employ a selective detection. In fact, preliminary studies were based mainly in spectroscopy UV absorption and fluorometric approaches due to the spectroscopic characteristics of some AGE-products. At present, common methods for the analysis of protein glycation products, both *in vivo* and *in vitro*, include immunochemical methods such as immunohistochemistry (Oya *et al.*, 1999; van Heijst *et al.*, 2005; Nagai *et al.*, 2008), enzyme-linked immunosorbent assay (ELISA) and Western blotting using available antibodies specific for certain AGE structures (Turk *et al.*, 2001; Ranjan *et al.*, 2006; Takeuchi *et al.*, 2010), or chromatographic techniques such as GC and HPLC coupled to mass spectrometry (Lapolla *et al.*, 2001; Thornalley *et al.*, 2003; Lapolla *et al.*, 2006; Cantero *et al.*, 2007), or HPLC coupled with fluorescence detection after protein hydrolysis (Usui *et al.*, 2004). Moreover, for separation, characterization and identification of these arising compounds, HPLC based approaches are the most frequently used, together with the fast analytical technique of capillary electrophoresis that is also applicable for the analysis of these compounds (Dutta and Dain, 2005). A flow chart that can be generally applied to the characterization of glycated species has been published by Lapolla et al. (Lapolla *et al.*, 1994) and is shown in figure 1.10.

Despite the different analytical strategies employed in the investigation of protein glycation, in some cases, the methodology lacks in specificity or involves too severe manipulation steps leading eventually to misleading results. Immunochemical methods are restricted to detection and relative quantitation of known and characterized marker compounds for protein glycation and are dependent on the different affinities of AGE epitopes for the antibodies, whereas the downside of chromatographic methods lies in high detection limits

as compared to low modification rates found especially *in vivo* studies. Furthermore, the requirement of a sufficient hydrolysis step that has to be mild enough to prevent artifact formation limits the use of GC or HPLC. With the application of mild ionization techniques such as ESI and MALDI-TOF, mass spectrometry has been employed in this field and its high specificity and sensitivity is very useful. Among the different mass spectrometric approaches, MALDI has been particularly important (Lapolla *et al.*, 1999), allowing studying intact proteins, without extended sample treatment which prevents the raising of possible artifacts.

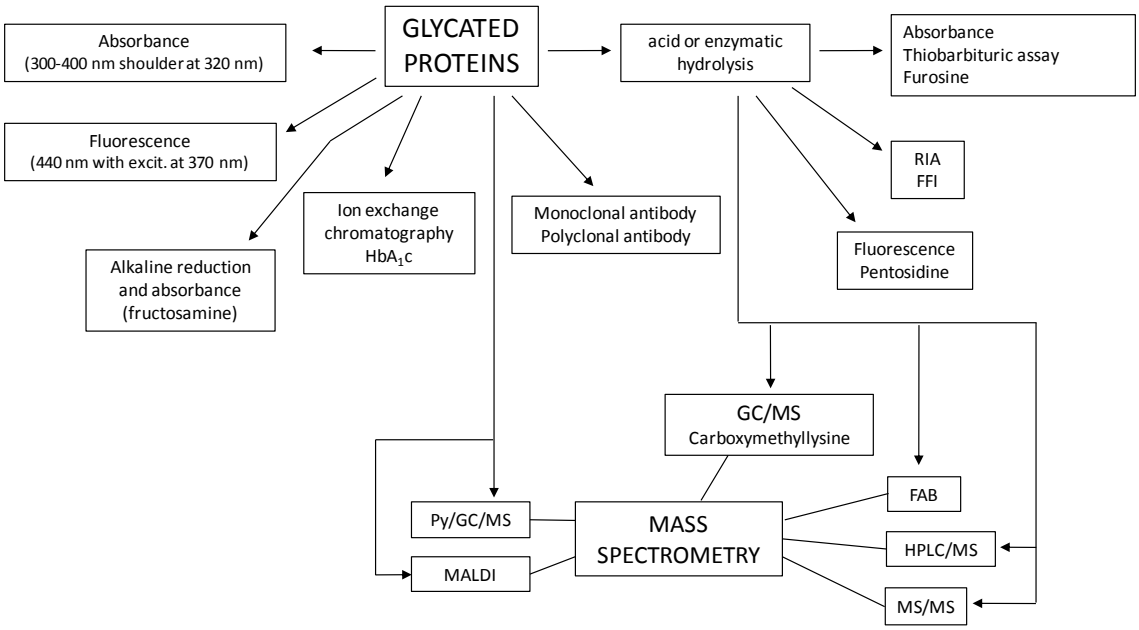

Figure 1.10 – Flowchart of analytical strategies for glycated protein analysis (Lapolla *et al.*, 1994).

Mass spectrometry in the study of protein glycation

Because of the great complexity of biological samples (plasma, cellular and subcellular fractions, etc.), direct identification of glycated proteins presents as a difficult task. Consequently, the majority of glycation studies have been

carried out by *in vitro* experiments in order to extrapolate the resulting information to the *in vivo* situation. Therefore, *in vitro* glycation of specific proteins or a mixture of them is usually carried out under pseudophysiological conditions. At the desired concentrations in a pH 7.5 buffer, the proteins are incubated with glucose concentrations from 10mM to 2M at 37ºC. The protein solution can also contain a bacteriostatic agent such as toluene or sodium azide and is usually maintained in anaerobic conditions. There are reports that focused on the biological buffers used in this type of investigation, which are mainly phosphate and bicarbonate buffers. The rate and selectivity of glycation is affected by the buffer (Shilton *et al.*, 1993), which should be taken in account when comparing *in vivo* and *in vitro* data. Other reports have focused on the incubation time which frequently ranges from 1 to 30 days, as well as on the glycation agent depending on the glycation level to be achieved (Ledesma-Osuna *et al.*, 2008). In the case of glyoxal and other compounds derived from glucose such as methylglyoxal, phenylglyoxal and diacetyl, the incubation time is usually shortened due to the higher reactivity of these compounds when compared to glucose (Knott *et al.*, 2003). The first experiments were performed by incubating BSA under pseudophysiological conditions with D-glucose at different concentrations and for different periods of time (Lapolla *et al.*, 1993). When incubated with glucose, BSA MALDI spectra showed a clear increase of molecular mass as a result of glucose adducts formation, an increase that depended on both sugar concentration and incubation time. Analogous investigations using MS-based approaches have been undertaken on bovine pancreatic ribonuclease (Lapolla *et al.*, 1994), lysozyme (Lapolla *et al.*, 1996), collagen (Paul *et al.*, 1998) and immunoglobulin G (Lapolla *et al.*, 2000). Data from these reports have shed light on the relationship between the glycation rate and the glycation agent, and more importantly have showed that mass spectrometry can be validly used for direct glycation level evaluation and for quantitative screening of the resulting advanced glycation products.

In the study of glycated proteins through MS-based approaches, the general workflow employed can be schemed in figure 1.11. Basically, there are three main steps: sample preparation, analysis by mass spectrometry and data processing. In most cases, separation techniques such as 1D or 2D gel electrophoresis, chromatofocusing, size exclusion and anion-exchange liquid

chromatography have been reported to study glycation in proteins but are not efficient in assessing glycation when proteins are present in a wide range of concentrations or are part of complex samples. Moreover, the information about the preferred glycation sites for each protein is lost during conventional enzymatic digestion of proteins. For these reasons, highly selective steps have to be undertaken both at the protein and peptide level. Methods based on affinity

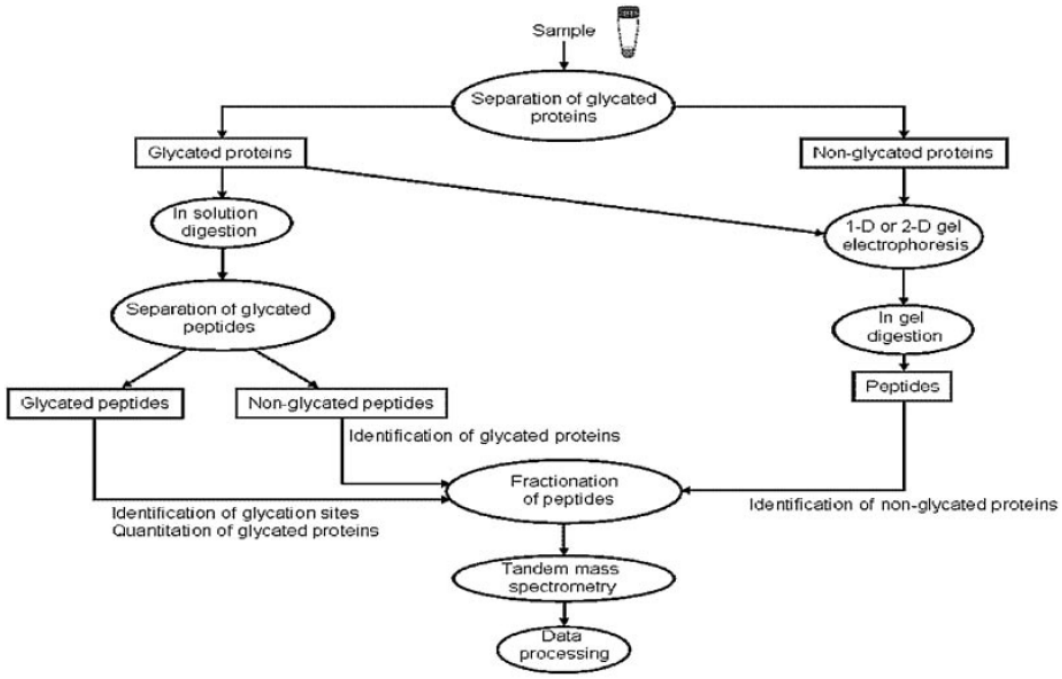

Figure 1.11 – Workflow for the mass spectrometry analysis of non-enzymatically glycated proteins (Capote and Sanchez, 2009).

chromatography using lectins and hydrazide supports have been used for selective separation of non-enzymatic glycated proteins (Monzo *et al.*, 2007). Boronic acids can also be used as ligands in affinity chromatography for purification of diverse biomolecules, including glycoproteins (Zhang *et al.*, 2007), which is known as boronate affinity chromatography (BAC). BAC is based on the interaction between boronic acids and cis-diol compounds that occurs through esterification under alkaline conditions, making it selective for non-enzymatically glycated proteins (Li *et al.*, 2002). Other secondary

interactions like hydrophobic interactions, ionic interactions, hydrogen bonding, and coordinated interactions can also play an important role in retention or elution because of steric or repulsion effects (Liu, 2006). These non-specific interactions could be responsible for the co-elution of both glycated and non-glycated proteins claimed by some authors. Nevertheless, BAC has been proved as a highly-efficient technique for isolation and enrichment of glycated proteins.

Following the isolation step, enzymatic digestion poses as a critical step in understanding the information concerning glycation sites. Protein modification by insertion of glucose molecules or derivatives may modify the digestion pattern. In addition, enzymatic digestion of glycated proteins is more difficult than that of non-glycated proteins. Enzymatic digestion is usually carried out in solution by conventional protocols involving high-quality enzymes after denaturation, reduction and alkylation, but can also be performed after gel-based electrophoresis procedures (Lapolla *et al.*, 2008). The application of multi-enzymatic treatments is a frequent approach to identify potential glycation sites by processing and intercrossing the information resultant from analysis of peptides generated by each enzyme. Because glycated lysines are protected by sugar moieties from digestion with lysine-specific enzymes (trypsin, endoproteinase Lys-C), other common enzyme employed is the highly selective endoproteinase Glu-C which cleaves peptide bonds C-terminally at every glutamic acid (Kislinger *et al.*, 2005). Due to the complexity of analyzing glycated proteins a fractionation step at peptide level has proved to be especially if coupled to MS and MS/MS detection. This coupling enables the increase of sensitivity, which is particularly interesting for identifying proteins in complex samples. Separation of peptides resulting from enzymatic digestion is usually carried out by liquid chromatography in C18 reversed phase, using gradient methods from formic acid aqueous solutions to acidified acetonitrile phases. Formic acid mobile phases are preferred in order to promote the ionization efficiency of glycated peptides in mass spectrometers (Gadgil *et al.*, 2007). In the characterization of glycation products, identification of predictable *in vivo* glycation sites and investigation of the fragmentation behavior of glycated peptides, liquid chromatography has been on-line connected to MS detectors with electrospray (ESI) or atmospheric pressure chemical ionization (APCI) methods, and also to matrix assisted laser desorption ionization (MALDI)

method usingrobot sampling devices (Lapolla *et al.*, 2004; Lapolla *et al.*, 2005; Cheng *et al.*, 2006; Frolov *et al.*, 2006). In addition to reversed phase separation, affinity chromatography with boronate phase has also been tested for enrichment of glycated peptides by its separation from non-glycated ones (Zhang *et al.*, 2007).

Recently, the performance of peptides separation on microfluidic chip-based devices has also being implemented in proteomic approaches to take benefit from advantages derived from the use of miniaturized devices. These microfluidic systems not only enable the accomplishment of peptides fractionation in shorter times but also represent a more versatile approach. They can provide the platform for enrichment columns for pre-concentration of peptides or clean-up from the sample matrix. Shmanai and co-workers have applied MALDI-MS analysis combined with affinity chromatography on microchips with immobilized phenylboronic acid-agarose gels for selective enrichment and detection of glycated proteins in plasma samples (Shmanai *et al.*, 2007). Physicochemical grafting of hydrophilic polymers on aluminum surface was developed to reduce nonspecific protein sorption and 3-aminophenylboronic acid (mPBA) was covalently immobilized as an affinity ligand to achieve specific binding of glycated plasma proteins. Glycated proteins were captured directly on the chip with high selectivity and efficacy with low nonspecific binding confirmed by MALDI-MS.

The initial studies on protein glycation were developed for quantification of the glycation level in specific proteins, where MALDI-MS (operating in linear, reflectron and post-source decay modes) provided interesting results both in *in vitro* and *in vivo* studies, associating glycation with pathological conditions such as diabetes (Lapolla *et al.*, 1993; Lapolla *et al.*, 1999). This estimation was based on the 162 Da mass shift per glycation site observed in glycated proteins, allowing to calculate the average number of glucose molecules attached to a protein. Thus, MALDI-MS has been used in the analysis of plasma proteins from diabetic uremic patients by determination of sugar residues attached to proteins such as serum albumin, immunoglobulins or globins. The main limitation is that this mass increase can be also observed for enzymatic O- and N-glycosylations and, therefore, conclusions should be considered with precaution. This type of studies has also been carried out by optical detectors and radioimmunoassay

(RIA) or ELISA approaches (Makita *et al.*, 1992; Zhang *et al.*, 2005). Nevertheless, these previous methodologies are not useful for analysis of low-concentrated proteins for which the application of peptide mapping approaches based on MS/MS is mandatory. Characteristic fragmentation patterns accompanying the parent and the fragment ions of glycated peptides from albumin studied by collision-induced fragmentation (CID) after ESI-MS or MALDI-MS have been identified (Frolov *et al.*, 2006). Neutral losses of 18, 36, 54, 72, and 84 u have been attributed to losses of water and formaldehyde. Furthermore, characteristic mass increments have been reported to correspond to AGEs such as CML (58u), pyrraline (108u), imidazolone A (144u), imidazolone B (142u), Schiff bases or Amadori products (162u), and 1-alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP) (270u) (Zhang *et al.*, 2003; Ahmad *et al.*, 2008). Precursor-ion scanning methods based on the Amadori-derived lysine immonium ion at a m/z 192.1 have been used to map glycation sites in albumin using ESI-QqTOF-MS instrument (Frolov *et al.*, 2006). However, this method has some limitations for broad applications in bottom-up proteomics, due to the low mass cut-off limitation of all commercial lower priced 3D or linear ion trap instruments (Zhang *et al.*, 2007). Moreover, when using collision-induced dissociation (CID) as the fragmentation mode, the spectrum of glycated peptides is marked by the high abundance of signals corresponding to various degrees of neutral water loss from the sugar moiety (Zhang *et al.*, 2007). During CID, intramolecular vibrational energy redistribution occurs prior to peptide fragmentation, which results in preferential dissociation of the weakest bonds on the monosaccharide and, therefore, in high abundance ions corresponding to various degrees of water loss. Although specific glycation sites have been identified from incubations of model proteins with glucose or other sugars, the assignation of glycation sites becomes a difficult task in complex samples.

A fragmentation method especially suited for the characterization of posttranslational modifications (PTMs) is the electron-transfer dissociation (ETD), which has recently been developed by Syka et al. (Syka *et al.*, 2004). This mode is similar to electron capture dissociation (ECD), implemented on Fourier transform ion cyclotron resonance mass spectrometers but with the electron source consisting of aromatic anions such as fluoranthene (Syka *et al.*, 2004). ETD fragmentation consists of bond dissociation immediately after electron

transfer, thus avoiding alteration of labile PTMs and providing complete sequence information as a result. Comparison between ETD and CID as fragmentation modes in the analysis of glycated peptides have been reported (Zhang *et al.*, 2007). Nevertheless, the downside of ETD is the lack of sensitivity in the analysis of less concentrated proteins due to the low efficiency of the process, which usually ranges from 20% to 30% as compared to that reported by CID (Zhang *et al.*, 2007). This can be problematic in the analysis of complex peptide mixtures where high-resolution FT-based MS is particularly efficient providing molecular weights of the glycated peptides at high mass accuracy (Marotta *et al.*, 2003). Another alternative is a method based on neutral loss scan (162 Da) by LC–QTOF-MS/MS recently developed for screening and sequencing of glycated proteins (Gadgil *et al.*, 2007). Firstly, all tryptic peptides eluted from the chromatographic column are subjected to low collision energy (5 eV) to prevent fragmentation. Then, the collision energy is increased to 20 eV to selectively promote the loss of 162 Da in the glycated peptides in the MS scan mode. If ions with this neutral loss are not found, the MS instrument returns to the low-energy MS scan mode to start the next cycle. Ions with neutral loss of 162 Da are selected in the quadrupole and subjected to MS/MS fragmentation at higher collision energy values to yield a series of *b*- or *y*-ions containing the peptide sequence information. Moreover, the mass spectrometer is set to monitor two ions simultaneously in the MS/MS step to enable the monitoring of two coeluting glycated peptides. This approach has provided good results in the study of *in vitro* glycation of HSA (Gadgil *et al.*, 2007). A possible interference should be taken into consideration due to the possible presence of proteins linked to mannose (either N- or O-linked) which are going to provide the same neutral loss of 162 Da.

1.2.3 Protein glycation and oxidative stress: the interrelationship

Although in all living organisms proteins are prone to a range of posttranslational non-enzymatic modifications, in the past years authors have considered oxidation and glycation as non-enzymatic modifications with particular interest. This interest arose from the possible interrelationship between these two posttranslational modifications in the genesis or as a consequence of biological events such as aging and health complications. On one hand, pro-oxidative species can causedirect damage to biological macromolecules or indirect damage through the generation of propagative species that spread the oxidative damage as well. On the other hand, non-enzymatic glycation appears as a complex multistep process, beginning with the binding of reducing sugars to free amino-groups of proteins, that eventually gives rise to so-called AGEs. At the time, hypotheses emerged with the suggestion of glycation and oxidation as two events strictly interconnected, in a synergism coined 'glycoxidation' (Baynes, 1991). Evaluation of the modification of rat collagen due to glycation and lipoperoxidation products through fluorescence measurements both *in vitro* and *in vivo* was reported by Odetti et al. (Odetti *et al.*, 1994). Results pointed to an increase of glycation- and oxidation-related fluorescence intensities with significant statistical correlation between their respective increments which, although not proving, suggested that oxidation and glycation processes could be *in vivo* mutually dependent. In addition, *in vitro* studies reported the effects of glycation on albumin previously exposed to oxidizing free radicals through γ-irradiation (Traverso *et al.*, 1996). Fluorescence alterations observed either in irradiated or in glycated proteins were similar and attributed to similar modifications in protein structure such as conformational changes, amino acid modifications, chain breaks and to the development of new fluorophores. Also, the effects of oxidation and glycation appeared to be additive, showing an interconnection between oxidation and glycation. Another study by Sobal and co-workers (Sobal *et al.*, 2000) reported the influence of glycation and oxidation in low-density lipoprotein (LDL), showing clearly that glycated LDL is more sensitive to oxidation than native LDL. The authors ascribed the fact that glycated LDL is more easily oxidized than

native LDL to its higher degree of AGE modification verified by AGE-specific fluorescence. The formation of free radicals during the glycation phase would explain the additional oxidative stress exerted by LDL-bound Amadori products and/or AGE-moieties observed in this study. Further evidence of the role of reactive oxygen species in the propagation of protein damage from glycation reactions is reported by Chetyrkin et al. (Chetyrkin *et al.*, 2008). In this paper, oxidative degradation of either glucose or protein–Amadori intermediates is shown to cause oxidative modification of protein tryptophan residues via hydroxyl radical, which in turn showed to affect protein function under physiologically relevant conditions. The influence of glycation was also studied on hemoglobin functional and structural properties (Sen *et al.*, 2005). The findings confirm glycated hemoglobin as an enhanced source of catalytic iron and that hemoglobin glycation also stimulated its autooxidation. Besides functional modification, glycation-induced structural modifications included reduced a-helix content, more surface accessible hydrophobic tryptophan residues, increased thermolability and weaker heme-globin linkage. The authors related these structural and functional modifications with pathophysiological complications of diabetes.

Following the evidences consistent with the hypothesis of the associated roles of glycation and oxidative stress in the alterations typical of aging and health sequaele, studies focused on investigating the consequences of glycoxidation products formation. The effects of glucose, low-molecular-weight carbonyls (such as methylglyoxal, glyoxal, glycoaldehyde and hydroxyacetone) and preglycated proteins on the activity of cellular enzymes (such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione reductase (GR) and lactate dehydrogenase (LDH)) were compared and the mechanisms of these processes were also investigated (Morgan *et al.*, 2002). It was shown that in all cases enzyme inhibition occurred and its extent was time- and concentration- dependent. Although the secondary damage inflicted by glycoxidized proteins or glycoxidation products on other biomolecules such as enzymes, lipids and DNA is though mainly to be radical-mediated (via free radical formation during protein glycation) (Yim *et al.*, 2000), this study of Morgan and co-workers showed that it can also result from a non-radical pathway via cross-linking or aggregation of the glycated protein with the target.

In an independent report, it is further demonstrated the evidence of cysteine proteases inactivation by reactive and protein-bound carbonyls with the formation of adduct species and crosslinks at the active thiol site (Zeng *et al.*, 2006). This study provided a possible explanation for the accumulation of modified proteins, particularly in lysosomes, and likely to occur in a wide range of cells exposed to oxidative and carbonyl stress typical of conditions such as diabetes and age-related pathologies.

Although it is acknowledged that AGE-proteins can be repaired by enzymatic mechanisms or removed by recognition with specific receptors, internalization and degradation by macrophages, lysosomes-containing cells or proteolytic enzymes (Schmidt *et al.*, 1994; Horiuchi *et al.*, 1996), cellular accumulation of oxidized and AGE-proteins is also a fact. Resistance of glycated proteins and severely oxidized proteins to degradation by the proteasome has been reported as one of the reasons (Bulteau *et al.*, 2001). Thus, the study of the deleterious consequences of AGE accumulation, as well as the mechanisms of inhibiting their formation or safely breaking them down and removing before they rise to the level of causing damage, constitute nowadays major topic lines of research.

1.3 Proteomics approach in the study of protein PTMs

In proteomics, most of the research is focused on determining protein expression levels as a function of cell normal growth or of perturbations induced by exogenous sources. However, cellular vital processes are not only governed by the relative abundance of proteins, but also by the regulation of the activity, association and localization of proteins and protein complexes. These properties are significantly determined by the tightly controlled and reversible PTMs of proteins. Hundreds of different types of PTMs have been characterized to date. Methods based on cDNA microarrays have revolutionized the field of mRNA-expression analysis but genomics or bioinformatics-based methods do not allow a detailed investigation of the structure and dynamics of

PTMs (Mata *et al.*, 2005). PTM consensus-site recognition and prediction algorithms based on DNA- and protein-sequence searches (Blom *et al.*, 2004) are generally not robust and tend to overestimate the number of modification sites in proteins compared with the actual PTM-site occupancy determined experimentally (Jensen, 2000). A unique feature of proteomics, a research field based on advanced protein chemistry, mass spectrometry and bioinformatics, is the ability to provide the means for detailed and global analysis of posttranslational modified proteins. Proteomics appears as a rapidly expanding field and the pace of its developmental relies on the integration of novel computational techniques, increasingly advanced and sensitive analytical methods, and optimized genetic or biochemical approaches for the study of protein structure, function, interactions and dynamics.

PTMs in proteins can be well characterized by MS with the following strategies: determination of intact molecular weight, mass fingerprinting of peptide mixtures derived by proteolytic digestion, chemical or enzymatic removal of the modification and sequencing of the modified peptides by tandem MS. PTMs are then associated with either an increase or a decrease in molecular mass. Therefore, MS represents an ideal tool for identification and characterization of PTMs in proteomic studies.

In 'classical' proteomics research, the method of choice for protein separation is two-dimensional gel electrophoresis (2D-PAGE), since it allows the simultaneous separation and visualization of thousands of proteins present in a given sample. Individual protein spots are visualized after gel staining or by autoradiography if proteins are pre-labelled. Proteins of interest are then excised, enzymatically in-gel digested and identified by mass spectrometry and sequence-database searching. Posttranslationally modified proteins can be selectively detected and visualized in the 2D gel. This is achieved by using a PTM-specific staining reagent or PTM-specific antibodies for western blotting, or by incorporating PTM-specific radiolabels into the proteins. For example, certain types of glycoprotein can be selectively stained or derivatized with fluorescent probes (Shin *et al.*, 2008), antiphosphotyrosine antibodies specifically detect phosphotyrosine-containing proteins (by western blotting) (Clarke *et al.*, 2008), and pulse labelling with ^{32}P helps in the selective detection of all types of phosphoproteins by autoradiography of the 2D gel (Aponte *et al.*, 2009). This

type of approach enables the global analysis of several classes of posttranslational modified proteins and the study of the overall status of PTMs of proteins in a given biological sample, as well as, the evaluation of the dynamics of PTMs as a function of development, differentiation and cellular responses to exogenous stimulus. A disadavantage associated with the use of 2D-PAGE is the undetection of low-abundance proteins (which is usually the case of posttranslationally modified proteins) that are under the dynamic range of the PTM-specific detection method or are masked by abundant co-migrating unmodified proteins.

The improvement of proteomics approaches relies on keys steps involving purification of organelles or protein species of interest prior to protein and posttranslational modifications characterization by tandem mass spectrometry analysis, enabling the sequencing of posttranslationally modified peptides using advanced features, such as data-dependent acquisition and multi-stage mass spectrometry. Until date, the most successful strategies employ various modification-specific enrichment steps or use combinations of protein digestion and peptide separation methods. These strategies aim to achieve high sensitivity and specificity and a wide dynamic range for the analysis of complex biological samples, so that low-abundance and low-stoichiometry PTMs are detected. Stages of modification-specific proteomics are exemplified in figure 1.12.

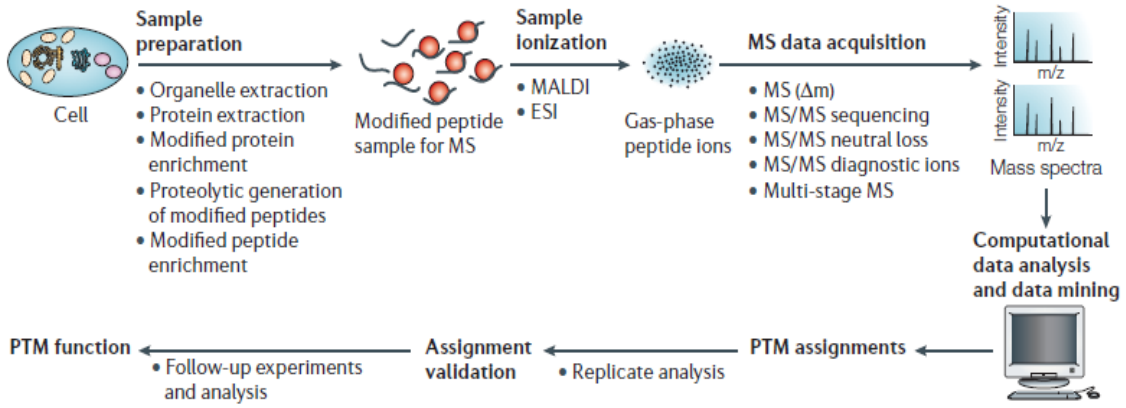

Figure 1.12 – Overall strategy of modification-specific proteomics (Jensen, 2006).

Usually, the analytical strategies combine PTM-specific affinity-enrichment techniques with electrophoretic and microfluidic separations, advanced mass spectrometry (MS) and bioinformatics (figure 1.12). PTM-enrichment techniques tipically rely on affinity or chemical approaches. When using affinity enrichment, selective separation or purification of modified proteins is achieved by immunoprecipitation using protein-specific antibodies. Individual proteins can be selectively purified, digested and then analysed by LC–MS/MS. For instance, phospho-specific antibodies are commercially available (for tyrosine, serine and threonine residues) and have been used in the investigation of cell signaling processes (Skalnikova *et al.*, 2008), in the enrichment of phosphorylated peptides in cancer proteomics studies (Gomase and Shyamkumar, 2009) and in the study of phosphoproteomes (Gronborg *et al.*, 2002). In the case of glycosylated proteins, lectins are considered as carbohydrate antibodies. Having distinct specificities towards various classes of glycans makes them suitable for enriching glycoproteins and glycopeptides (Calvano *et al.*, 2008). Various types of lectins can be combined, allowing improving the selectivity and specificity of proteomics experiments that aim to investigate N-glycosylated and O-glycosylated proteins and peptides. PTM-enrichment techniques are further improved by complementing affinity-based approaches with various chemical methods for the specific capture, immobilization and alteration of posttranslationally modified proteins/peptides to improve the overall dynamic range, sensitivity and specificity of the analysis. Solid-phase extraction provides a support for carrying out chemical and biochemical reactions that are followed by selective release and analysis of posttranslationally modified peptides by mass spectrometry. It is the case of O-phosphorylated or O-glycosylated serine and threonine residues, which are typically converted to more tractable species by using β-elimination and Michael addition reactions (McLachlin and Chait, 2003). Fluorous affinity tags have also been used as an approach for the enrichment of posttranslationally modified peptides, like phosphoserine peptides, by converting them into perfluorinated species that can be efficiently recovered by solid-phase extraction and analyzed by mass spectrometry (Brittain *et al.*, 2005). Tagging approaches have emerged as attractive and effective techniques in the field of modification-specific proteomics. Various stable-isotope labeling (SIL) techniques have contributed in the improvement of efficiency, accuracy,

throughput and sequence coverage of MS-based protein posttranslational modifications analysis and quantification. There are two main strategies that rely on *in vitro* SIL of amino acid residues: isotope-coded affinity tag (ICAT) through cysteine residues labeling and isobaric tag for relative and absolute quantitation (iTRAQ) through labeling of C-termini of cleaved peptides with ^{18}O and/or labeling of amino groups (N-terminal and lysine side chains). An example of the ICAT approach is a recent report by Fedorova and co-workers (Fedorova *et al.*, 2010), where the effects of ROS on actin were investigated in an established rat model. Among other parameters, such as polymerization ability and ATPase activity, oxidized amino acid residues such as cysteine, methionine and tryptophan were studied through blotting techniques and relatively quantified by MALDI and ESI mass spectrometry. Recently, also applied in investigating protein oxidative damage, isobaric tagging for relative and absolute quantification (iTRAQ) combined with LC-MALDI-MS/MS was employed in a proteomic approach for the identification of oxidative changes in hemoglobin and hemoglobin-haptoglobin complexes when subjected to H_2O_2 (Pimenova *et al.*, 2010).

Additionally, through metabolic culturing, particular types of amino acid residues containing stable isotopes (for example, ^{15}N and ^{13}C) can be used as mass tags that are incorporated biosynthetically into cellular proteins *in vivo*. This is known as SILAC, stable-isotope labeling by amino acids in cell culture, a MS-based tagging approach that allows quantitative proteomics studies to be carried out in a range of eukaryotic cell lines (Ong *et al.*, 2002; Bonenfant *et al.*, 2007). The combination of SILAC and MS has been successfully used to investigate PTM-dependent temporal and functional aspects of histones during cell cycle (Bonenfant *et al.*, 2007).

1.4 Aims and model proteins

At the present, the fact that most proteins undergo PTMs, either by proteolytic cleavage or by addition of a modifying group to one or more amino acids, represents a major topic of research. The increase on the knowledge of these modifications is of extreme importance, since these modifications are able to alter physical and chemical properties, folding, conformation, distribution, stability, activity, and ultimately, protein function. Among the most studied PTMs are protein non-enzymatic glycation and protein oxidation. Effort has been made in the study of these PTMs in relation to specific disease conditions with the aim of understanding the underlying events and proposing possible outcomes for prevention and/or treatment. This has prompted a great deal of research using proteomic approaches owing to developments in the field of mass spectrometry. Mass spectrometry has had a profound impact on addressing structural problems and, with the introduction of 'soft ionization' techniques like electrospray ionization and matrix assisted laser desorption ionization, valuable information about the primary and higher orders of protein structures along with their modifications can be provided. Thus, mass spectrometry not only enables protein characterization but also represents the candidate reference method for protein modifications analysis due to the specific mass changes that occur with relation to the molecular weight expected in the correspondent native protein.

In the present study we attempted to characterize the oxidative modifications found in purified forms of four different proteins subjected to *in vitro* oxidative stress, before and after induction of non-enzymatic glycation, using a mass spectrometry platform. The proteins chosen as models differ from each other in molecular weight, structure and amino acid composition. We initiated our investigation with BSA, an important plasma protein with antioxidant capacities that serve to protect key cellular and regulatory proteins. Being oxidative modifications of albumin responsible for altered biochemical properties, our goal was to study the structural changes resulting from oxidative stress, using a mass spectrometry based approach (MALDI-MS and MALDI-MS/MS) combined with off-line nano liquid chromatography. The following

protein was insulin which is an important protein with respect to its involvement in glucose uptake, as well as, to the alterations in its functionality found in hyperglycemic pathological conditions. So, we propose to structurally characterize glycated insulin through an approach employing HPLC purification of insulin glycated forms, followed by enzymatic digestion and MALDI-TOF/TOF analysis for the identification of glycation sites. We further investigated the oxidative behavior of insulin both in its native form and after non-enzymatic glycosylation for the determination and comparison of the oxidative damage. A similar methodology was engaged for the study of two histone proteins, specifically histone H2B and histone H1. Histones are known to be highly susceptible to non-enzymatic glycation and the most susceptible histones to this PTM are histone H1 and the dimmers H2A/H2B even when assembled in the nucleosome. Given that histones represent a protective barrier for DNA against external damage such as oxidative and/or glycoxidative damage, the study of the alterations caused by this type of damage appears as a relevant task. We intended to study histones H1 and H2B 'glycoxidative' damage by investigating the structural changes occurring in pre-glycated histones exposed to oxidative damage.

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2. Characterization of BSA oxidative modifications

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RCM

Oxidation of bovine serum albumin: identification of oxidation products and structural modifications

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Albumin is an important plasma antioxidant protein, contributing to protecting mechanisms of cellular and regulatory long-lived proteins. The metal-catalyzed oxidation (MCO) of proteins plays an important role during oxidative stress. In this study, we examine the oxidative modification of albumin using an MCO *in vitro* system. Mass spectrometry, combined with off-line nano-liquid chromatography, was used to identify modifications in amino acid residues. We have found 106 different residues oxidatively damaged, being the main oxidized residues lysines, cysteines, arginines, prolines, histidines and tyrosines. Besides protein hydroxyl derivatives and oxygen additions, we detected other modifications such as deamidations, carbamylations and specific amino acid oxidative modifications. The oxidative damage preferentially affects particular subdomains of the protein at different time-points. Results suggest the oxidative damage occurs first in exposed regions near cysteine disulfide bridges with residues like methionine, tryptophan, lysine, arginine, tyrosine and proline appearing as oxidatively modified. The damage extended afterwards with further oxidation of cysteine residues involved in disulfide bridges and other residues like histidine, phenylalanine and aspartic acid. The time-course evaluation also shows the number of oxidized residues does not increase linearly, suggesting that oxidative unfolding of albumin occurs through a step-ladder mechanism. Copyright © 2009 John Wiley & Sons, Ltd.

Protein oxidation has become in the past few years an even greater focus of research, since increased levels of oxidized proteins are one of the markers of oxidative stress. It is almost unquestionable that posttranslational modifications (PTMs) caused by oxidative damage from reactive oxygen and nitrogen species on protein integrity is responsible for many pathologies and biological aging (reviewed by Beal[1]). However, there is a significant difficulty in identifying important PTMs through *in vivo* approaches because of protein turnover and repair events.[2] Another reason is related to the potential chemical instability of these oxidative modifications. *In vitro* oxidation experiments have made important contributions to mechanistic studies and in identifying 'novel' protein oxidation products.[3–11] Nowadays, mass spectrometry (MS) has become the technique of choice for the qualitative detection of protein PTMs. In fact, it allows an unambiguous assignment of the nature and location of the change within a protein sequence. In the 1990s, Finch and co-workers identified modified residues in human serum albumin (HSA) when oxidized with hydrogen peroxide.[12] These authors identified, using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS), five sites of oxidation, including one cysteine and four methionines. More recently, several independent reports have focused on identifying specific oxidation targets under conditions of metal-catalyzed oxidation (MCO), recognized as a site-specific mechanistic reaction. For example, two histidines, His-13 and His-14, were found to be target residues in the human and mouse β-amyloid peptide (β-AP) under conditions of site-specific MCO.[2,6,7] Also, Bridgewater and co-workers,[3] in a study of oxidized human angiotensin I and bacitracin, show that known metal-binding amino acid residues are the preferred targets for oxidation of both proteins. Shortly after, in a MCO study of α-synuclein, the most susceptible residue to oxidation was found to be methionine.[13] A recent paper by Deterding and co-workers[14] reinforces the utility of both top-down and bottom-up MS-based approaches to study protein-free radicals. The precise amino acid residues (i.e., radical site) were identified through MS-based sequencing and Deterding and co-workers identified the specific location of DMPO adducts on several heme-containing proteins.[15,16]

Recently, two separate reports have described an approach based on a biotin hydrazide labelling method coupled with avidin affinity chromatography for identification of carbonyl groups in oxidized proteins.[11,17] Mirzaei and Regnier[17] identified proteins with one or two oxidations sites, mainly at arginine and methionine, but also at lysine and proline residues. In the other manuscript,[11] after subjecting HSA to model oxidative stress, the authors found essentially lysine residues oxidized to 2-aminoadipic semialdehyde. This study identified, either using MCO or treatment with

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hypochlorous acid (HOCl), seven different oxidized lysine residues.

In the present work, we describe a MS analysis of bovine serum albumin (BSA) subjected to *in vitro* oxidative stress. BSA is a 66 kDa protein of 607 amino acids in the precursor form (SwissProt P02769) and 583 amino acids in the secreted form, which lacks the first 24 residues. The chemical sequence of BSA is essentially the work of Brown and co-workers,[18,19] and their latest sequence[20] has been confirmed by cDNA sequencing, MS and homology with other albumin sequences. The secondary structure of BSA includes 67% helix, 10% turn, and 23% extended chain,[21] with 17 disulfide bridges supporting a triple-domain configuration with nine loops arranged in the order long-short-long. BSA is known to be one of the most important plasma antioxidants in protecting key cellular and regulatory proteins.[22] This antioxidant capacity correlates with the large quantity and high turnover of BSA, as well as the high reactivity of BSA sulfhydryl groups with oxidant species. Oxidative modifications of albumin are responsible for different biological properties, the study of these changes being a topic of great interest. To gain insights into the structural changes, we analyzed tryptic digests of oxidized BSA using different conditions and a MS-based approach (matrix-assisted laser desoprption/ionization (MALDI)-MS and MALDI-MS/MS) combined previously with off-line nano-LC. This approach allowed us to identify specific sites of oxidative modification, as well as the time-course evaluation of these changes on albumin.

EXPERIMENTAL

Chemicals and reagents

BSA (A6003, fatty acid free), trifluoroacetic acid (TFA) and EDTA were acquired from Sigma (St. Louis, MO, USA). $FeCl_2$ and hydrogen peroxide used for the oxidation reaction were acquired from Merck (Darmstadt, Germany). The MALDI matrices, α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid, were also supplied by Merck (Darmstadt, Germany). The organic solvents such as methanol, toluene and acetonitrile (ACN) were from Merck (Darmstadt, Germany), Riedel-deHaën (Buchs, Switzerland) and Labscan (Dublin, Ireland), respectively.

All chemicals were of analytical grade and milli-Q water was used throughout all experiments.

BSA *in vitro* oxidation

All oxidation reactions were performed by incubating samples in a water bath at 37°C. Fenton oxidant was used for metal-catalyzed oxidation (MCO) experiments. According to Baron *et al.*,[23] $FeCl_2$, EDTA and hydrogen peroxide were added in the oxidative mixture, using a ratio of 1:1 of $FeCl_2$/EDTA because it has pro-oxidative effects.

Two oxidation conditions, Ox_2 and Ox_10, were used. A typical oxidation reaction contained, in 10 mM phosphate buffer (pH 7.4), 0.05 mM BSA and 0.05 mM $FeCl_2$/EDTA and 0.1 mM H_2O_2, in the case of Ox_2 conditions. For Ox_10 conditions, Fenton oxidant concentrations were 0.25 mM $FeCl_2$/EDTA and 0.5 mM H_2O_2. After various incubation periods (0.5–24 h), reaction was stopped by acidification with 1% TFA and subsequentially frozen in liquid N_2, unless used for further analysis. All samples were stored when necessary at −80°C. Control reactions were performed simultaneously by incubating BSA alone in phosphate buffer. All experiments were performed in triplicate.

Protein carbonyl groups

Protein carbonyl content was determined using 2,4-dinitrophenylhydrazine (DNPH), as previously described by Levine *et al.*[24] and Rajesh *et al.*,[25] using slight adjustments.

Previously to the assay, oxidized samples were filtered through Ultrafree-MC micro-centrifuge filters (nominal molecular weight cut-off 5000 Da; Sigma), at 5000 *g* for 20 min. After the first centrifugation, an equal volume of milli-Q water was added and the solution centrifuged again. The resulting pellet was resuspended in phosphate buffer and stored until further analysis.

Briefly, samples (40 μg) were incubated in the dark (room temperature) for 1 h with 160 μL of 10 mM DNPH prepared in 2.5 M HCl. Controls were prepared by incubation in 2.5 M HCl alone. All assays were performed in triplicate. After the incubation, samples were washed twice with TCA (10 and 20%) and the supernatant discarded. Finally, the protein pellets were washed al least three times with 160 μL of ethanol/ethyl acetate (1:1, v/v) to remove unreacted DNPH. The final protein pellet was dissolved in 500 μL of 6 M guanidine hydrochloride and incubated at 37°C for 10 min. For determining protein concentration, using the RC DC Protein Assay kit (Bio-Rad), a parallel assay was performed. The carbonyl content was calculated using an absorption coefficient of 22 000 $M^{-1} cm^{-1}$ ($\lambda = 370$ nm).

Gel electrophoresis

Modifications of BSA after *in vitro* oxidation were analyzed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. To ensure total protein solubilization, we used a solution of urea (8 M) and thiourea (2 M) before gel electrophoresis. Samples were subsequently treated with 10% SDS, 0.5 M Tris pH 6.8, 15% glycerol and 2.9 M 2-mercaptoethanol and resolved by 12.5% SDS-PAGE according to Laemmli's method. The gels were stained either with Coomassie blue[26] or with imidazole-zinc negative staining.[27] For image analysis each gel image was acquired using the calibrated imaging Gel-Doc (Bio-Rad) and analyzed with the Quantity One v4.6 software (Bio-Rad). The software allowed background subtraction, automatic band detection and comparative analysis of normalised band optical densities (ODs). The imidazole-zinc staining was used to avoid interference events, caused by the oxidation of basic and aromatic amino acid chains and thus decreased dye binding ability with Coomassie staining.[28] Therefore, we show the results using the approach with the negative imidazole-zinc staining.

Enzymatic digestion, nano-reversed-phase high-performance liquid chromatography (RP-HPLC) and collection of MALDI fractions

For in-solution tryptic digestion oxidized BSA samples were initially diluted in 25 mM ammonium hydrogen carbonate buffer. Trypsin solution (Promega, Madison, WI, USA) was

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added in a final ratio of substrate/enzyme of 40:1 (w/w). Samples were incubated overnight at 37°C and after the incubation period tryptic digests were dried under vacuum in a Speed-vac (ThermoSavant, Holbrook, NY, USA).

For direct application onto MALDI plates, tryptic digests were redissolved in 50% ACN/0.3% TFA and mixed (1:1) with a solution of 5 mg/mL of α-CHCA prepared in 50% ACN/0.3% TFA or with a solution of 10 mg/mL of 2.5-hydroxybenzoic acid (DHB) prepared in 50% ACN/0.1% TFA. The method for application with the DHB matrix was the dried-droplet method.[29] Aliquots of 0.5 μL were loaded on an MALDI plate.

Tryptic digests of oxidized BSA samples were separated by LC using an Ultimate 3000 column (LC Packings). For each sample, 1–3 μg of total protein were injected. First, samples were injected onto a C18 trapping column (Zorbax 300SB-C18, 5 μm particle size, 5 × 0.3 mm; Agilent Technologies) using an autosampler. Samples were washed over the trapping column for 3 min isocratically with 95% buffer A (water, 0.1% TFA) and 5% buffer B (80% ACN, 0.04% TFA) at a flow rate of 30 μL/min. After 3 min, the flow was redirected to the analytical column (Zorbax 300SB capillary analytical C18 column, 3.5 μm particle size, 150 mm × 75 μm; Agilent Technologies) at a flow rate of 0.3 μL/min. Separation of tryptic peptides occurred using a linear gradient of 5–15% buffer B over 10 min, 15–50% buffer B over 47 min, 50–90% buffer B over 2 min, followed by a 3-min hold at 90% buffer B. The peptides eluted from the monolithic capillary column were immediately deposited onto 384-well MALDI plates using a Probot (LC Packings), with 20-s intervals for each spot with simultaneous application of 170 nL of α-CHCA matrix solution. α-CHCA matrix solution was prepared by diluting saturated α-CHCA with 70% ACN/0.3% TFA and adding 10 fmol of Glu-Fib as internal standard.

Mass spectrometry and database search

Peptide mass spectra were obtained with a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were acquired in the mass range between 800 and 4500 Da with ca. 1500 laser shots. For the following acquisition of tandem mass (MS/MS) spectra of each sample spot, a data-dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis or acrylamide peaks. Trypsin autolysis, Glu-Fib and albumin theoretical tryptic masses were used for internal calibration of mass spectra, allowing a routine mass accuracy better than 20 ppm.

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems) which uses internal Mascot (Matrix Science) software to search peptide mass fingerprints and MS/MS data. Up to two missed cleavage sites were allowed and for the search for oxidized peptides the Mascot software was updated through the UNIMOD website.[30,31] Oxidative-related modifications were selected from the modifications listed and available in the UNIMOD website[30] and set differentially for each possible modified residue. Each modification in each residue (e.g., oxidation [Cys]) represents one possible choice for a database search. The database searches were performed using MS/MS data for each modification listed in Table 1, using the option of 'variable modifications'. This option was chosen because it allows finding matching peptides that are both modified and unmodified by oxidative damage and because it allows investigation of more than one modification in the same residue within the same database search. In a first approach, for each modification, searches were performed separately for each residue, since there are modifications (oxidation, deamidation, carbamylation) that are possible to occur in more than one residue. In a second phase, different modifications in different residues were combined within the same database search. No more than four variable modifications were combined in a single database search in order to prevent an increase in the number of false positive identifications. So, positive identifications for oxidatively modified peptides were considered for individual ion scores above 40 with the default significance threshold, $p < 0.05$. All modification sites were manually validated to obtain both the

Table 1. List of oxidative modifications updated from the UNIMOD website[30] through the mod_file of Mascot software

Modification	Description	Δm (monoisotopic)
Amino (Tyr)	Tyrosine oxidation to 2-aminotyrosine	15.0108
Arg → GluSA	Arginine oxidation to glutamic semialdehyde	−43.0534
Cys → Oxoalanine	Cysteine oxidation to oxoalanine	−17.9928
His → Asn	Histidine oxidation to aspargine	−23.0159
His → Asp	Histidine oxidation to aspartic acid	−22.0319
Lys → Allysine	Lysine oxidation to aminoadipic semialdehyde	−1.0316
Lys → Aminoadipic acid	Lysine oxidation to α-aminoadipic acid	14.9632
Pro → Pyrrolidone	Proline oxidation to pyrrolidone	−27.9949
Pro → Pyrrolidinone	Proline oxidation to pyrrolidinone	−30.0105
Pro → Pyro-Glu	Proline oxidation to pyroglutamic acid	13.9792
Trp → Hydroxykynurenin	Tryptofan oxidation to hydroxykynurenin	19.9898
Trp → Kynurenin	Tryptofan oxidation to Kynurenin	3.9949
Trp → Oxolactone	Tryptofan oxidation to oxolactone	13.9792
Trioxidation (Cys)	Cysteine oxidation to cysteic acid	47.9847
Oxidation, and dioxidation	Oxygen addition and hydroxylation (Lys, Arg, Cys, Met, Tyr, His, Pro, Trp, Phe, Asp, Asn)	31.9898
Deamidation (Arg, Asn, Gln)		0.9840
Carbamylation (Lys, Arg, Cys, Met)	Isocyanate reaction with amino groups	43.0058
Quinone (Tyr)		29.9741

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correct peptide sequence and exact modification site. Matched sequences were validated if all major peaks in the MS/MS spectrum were explained by the candidate sequence and the spectrum contained peaks to confirm the peptide's modification.

RESULTS AND DISCUSSION

In this study, we examine the oxidative modification of albumin using an MCO *in vitro* system. Two oxidation conditions, Ox_2 and Ox_10, were used and samples were analyzed for different periods of time (0–12 h). First, changes in protein carbonylation were detected as these are widely used as an important marker of protein oxidation.[1,32,33] Our results show that oxidation of BSA leads to a significant increase in carbonyl groups (see Fig. 1). The *in vitro* oxidation of BSA produces as much as 3.6 μmol (12 h of oxidation with Ox_10 conditions) of bound carbonyls per mg of protein. Results show the different concentrations of oxidant agent lead nearly to the same carbonyl indices after 12 h of oxidation. Bound carbonyls increase exponentially for longer oxidation periods, which agrees with results from Traverso and co-workers.[34] Major differences are seen along the time-course of the oxidation reaction: higher hydrogen peroxide concentrations leads to more rapid carbonyl formation. Carbonyl group formation results from both oxidation of amino acid side chains and cleavage of the polypeptide backbone, which in turn is responsible for protein cross-linking events.[32] Therefore, we studied changes in oxidized BSA samples by SDS-PAGE in reducing conditions and with Coomasssie blue and imidazole-zinc gel staining. In Fig. 2, it is possible to see, for longer oxidation periods, a progressive fading of the native BSA band, which is compatible with the carbonyls data. This fading corresponds to a decrease of 15% with the highest concentration of hydrogen peroxide (Ox_10 conditions) and after 2 h of oxidation. The decrease is higher between 1 and 2 h of oxidation, rather than progressing linearly along the time-course of the oxidative damage. It was not possible to detect low molecular weight oxidation products that would explain the native BSA band

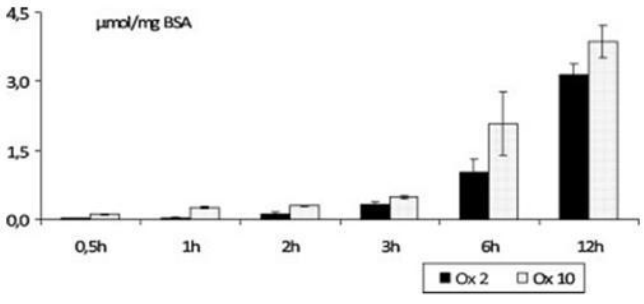

Figure 1. Content of protein carbonyl groups on BSA (0.05 mM) after incubation with different concentrations of $FeCl_2$/EDTA/H_2O_2 at 37°C in phosphate buffer (10 mM, pH 7.4). The average final values presented for oxidized samples were obtained after subtracting the corresponding control reaction values. Ox_2 – 0.05 mM $FeCl_2$/EDTA and 0.1 mM H_2O_2; Ox_10 – 0.25 mM $FeCl_2$/EDTA and 0.5 mM H_2O_2.

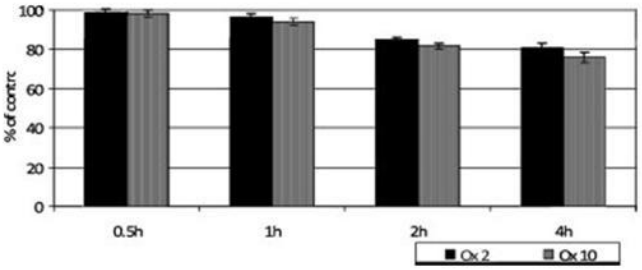

Figure 2. Reduction in optical density of BSA band (10 μg) of samples subjected to *in vitro* oxidation (BSA, 0.05 mM) for a total of 4 h with different concentrations of oxidative agent. Before gel electrophoresis oxidized samples were mixed with a solution of urea/thiourea (8 M/2 M) to ensure protein solubilization. Comparative values are shown in percentage relative to control samples. Ox_2 – 0.05 mM $FeCl_2$/EDTA and 0.1 mM H_2O_2; Ox_10 – 0.25 mM $FeCl_2$/EDTA and 0.5 mM H_2O_2.

fading. Nevertheless, the decrease in the original BSA band intensity suggests a loss of the native protein through modifications induced by oxidation related to protein fragmentation and/or precipitation.

The next step of our work focused on discovering BSA changes using a MS-based approach. The two oxidation conditions, Ox_2 and Ox_10, were analyzed for different periods of time (0, 30, 60 and 120 min). LC was used to separate the tryptic digests of oxidized and nonoxidized BSA. Resultant fractions were analyzed using an MALDI-TOF/TOF mass spectrometer for peptide sequencing and identification through tandem mass spectrometry. Typical chromatograms show a similar profile between control and oxidized samples (results not shown). Database searches for oxidation peptides were performed with Mascot ($p < 0.05$) using modifications defined on the Unimod website.[30] The modifications, shown in Table 1, were searched individually for each possible residue. To detect all possible modified residues, oxidized samples were not derivatized during the analysis process. In addition, to avoid producing 'false' modifications, during solubilization of oxidized samples, urea or detergents were not used. This approach results in 81% of protein coverage and allows identification of 106 modified residues in oxidized BSA after 2 h of oxidative damage. Oxidative-related modifications were only found in the case of samples submitted to 30 min, 1 and 2 h of oxidative damage (Ox_2 and Ox_10) and are available as Supporting Information (Supplementary Table 1). For each modification, Supplementary Table 1 lists the peptide sequence, locating the residue within the sequence, as well as the observed masses for each peptide sequence with the associated error. Figure 3 shows two examples of modified peptides. In the case of Fig. 3(A), identifying ions belonging to the *y* fragmentation series allows location of the residue to Cys-58. In the MS/MS spectrum of the precursor ion at *m/z* 1769.76, it is possible to see the mass difference of 47.9 Da between the ion at *m/z* 1022.7 (*y*8) and the ion at *m/z* 871.4 (*y*7). This gap corresponds to the loss of the cysteine residue as cysteic acid. In the case of Fig. 3(B), identifying ions belonging to the *b* fragmentation series of the ion at *m/z*

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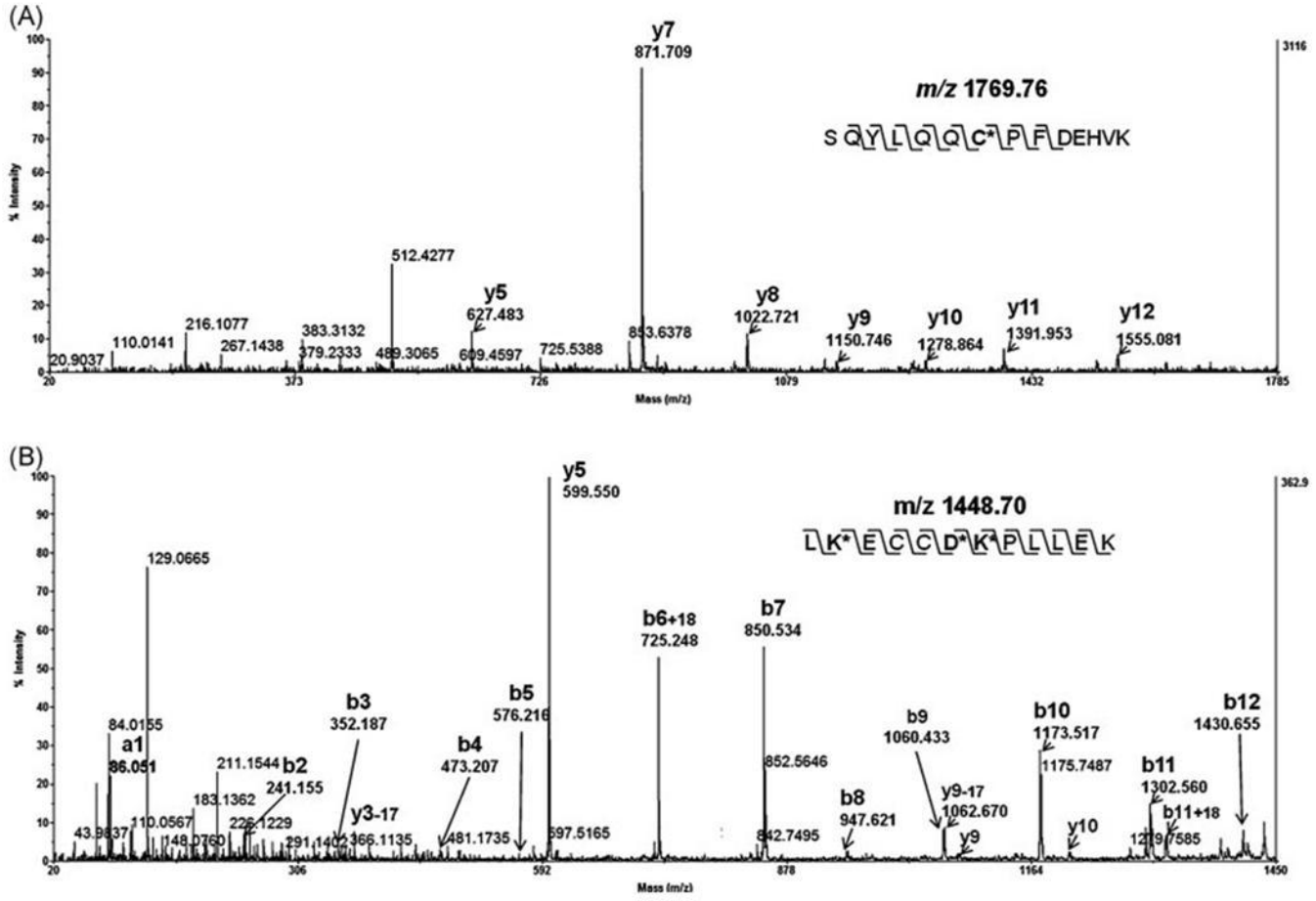

Figure 3. MS/MS spectra of the oxidatively modified peptides at *m/z* 1769.76 and 1448.70. The peptides corresponding to (A) the sequence between AA 52-65, that confirms Cys-58 is converted into cysteic acid with an increment of 47.9 Da, and (B) the sequence between AA 298–309 modified in three different residues: Lys-299 is oxidatively converted into allysine, Lys-304 is converted into aminoadipic acid and Asp-303 is oxidized.

1448.70 locates the three modified residues in the sequence between AA 298-309: Lys-299 oxidatively converted into allysine, Lys-304 converted into aminoadipic acid and oxidized Asp-303.

Even though all amino acid residues of proteins are susceptible to oxidation, the main affected residues observed in our study were cysteine, methionine, tryptophan, lysine, arginine, histidine, tyrosine and proline. This agrees with the literature since these are amino acids specially prone to oxidative damage.[35] It is known that MCO systems readily oxidize the side chains of these amino acids.[36] In Fig. 4(A), it is possible to see the different types of oxidative modifications found for each residue, for Ox_2 samples after 30 min and 2 h of oxidation. We also can see that the residues that are initially oxidized are mainly cysteine, methionine, lysine, arginine, proline and tyrosine. After 2 h, we observe oxidative damage affecting not only abundant residues such as cysteines, lysines and arginines, but also residues such as tyrosine, histidine, phenylalanine and aspartic acid. As expected,[32,36–38] oxidized side chains of lysine, arginine and proline residues also yielded carbonyl derivatives, such as aminoadipic semialdehyde and α-aminoadipic acid (Lys), glutamic semialdehyde (Arg) and pyrrolidinone (Pro). We also found cysteine oxidation to cysteic acid,[32] histidine oxidation to aspartic and asparagine residues,[32] and tyrosine oxidation to aminotyrosine and hydroxyphenylalanine (quinone).[10,32] Besides these expected modifications, we were able to detect carbamylation in oxidized lysine residues (and in cysteine and methionine) and lactone formation from an oxidized tryptophan residue. Carbamylation is a posttranslational modification of proteins, characterized by binding of isocyanate to amino groups. Isocyanate is generated from protein fragmentation[39] and binds to amino and sulfhydryl groups[40] of lysine, arginine, cysteine and methionine residues. Thus this modification is considered a product of secondary oxidative reactions.

One of the objectives of this work is to perform a time-course evaluation of the oxidative modifications of albumin, considering the oxidation rate of any side chain is influenced by solvent accessibility and inherent chemical reactivity of the side chain.[41] Different regions of the protein are affected at different time-points. Although the secreted form of BSA lacks the first 24 residues, all through the discussion we will employ residue numbers from the precursor form. According to the amino acid sequence proposed by Brown,[42] BSA comprises three homologous domains (I, II and III) that subdivide into three subdomains, namely a–b and c (see Fig. 4(B)).

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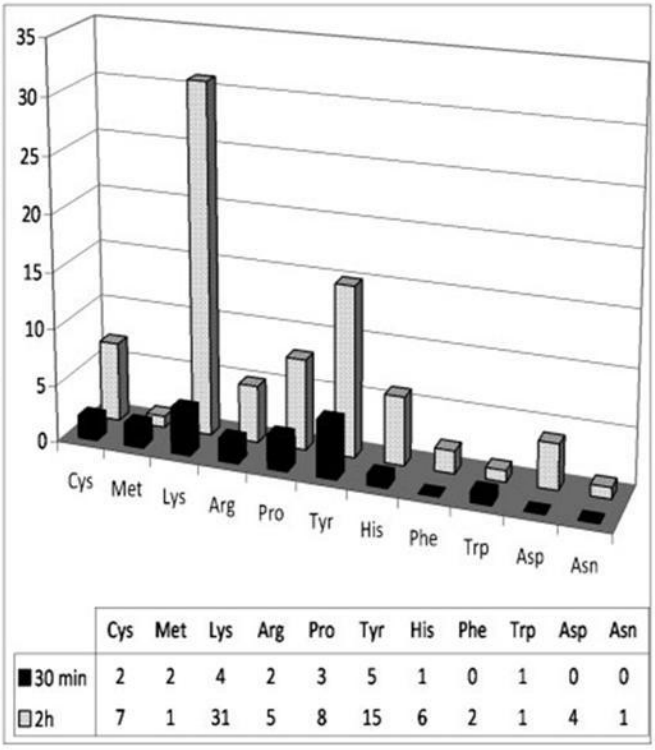
(A)

Modifications/ Residues	Ox_2	
	30 min	2h
Alpha-amino adipic acid (Lys)	1	6
Amino (Tyr)	1	4
Aminoadipic semialdehyde (Lys)	2	15
Aspargine conversion (His)	-	1
Aspartic acid conversion (His)	-	2
Carbamyl (Cys, Met, Lys, Arg)	1 (Cys) 1 (Met)	2 (Lys) -
Deamidation (Arg, Asn, Gln)	1 (Arg)	-
Dioxidation (Lys, Arg, Cys, Met, Tyr, His, Pro, Trp, Phe, Asp, Asn)	1 (Cys) 2 (Pro) 1 (Tyr)	3 (Cys) 5 (Lys) 2 (Pro) 2 (Tyr)
Glutamic semialdehyde (Arg)	1	1
Oxidation (Lys, Arg, Cys, Met, Tyr, His, Pro, Trp, Phe, Asp, Asn)	1 (Met) 1 (Lys) 1 (Pro) 2 (Tyr) 1 (His)	3 (Cys) 1 (Met) 3 (Lys) 4 (Arg) 3 (Pro) 6 (Tyr) 3 (His) 2 (Phe) 4 (Asp) 1 (Asn)
Oxoalanine (Cys)	-	1
Oxolactone (Trp)	1	1
pyro-Glutamic acid (Pro)	-	1
Pyrrolidinone (Pro)	-	2
Quinone (hydroxyphenylalanine) (Tyr)	1	3
Trioxidation (Cys)	1	3



	Cys	Met	Lys	Arg	Pro	Tyr	His	Phe	Trp	Asp	Asn
30 min	2	2	4	2	3	5	1	0	1	0	0
2h	7	1	31	5	8	15	6	2	1	4	1

Figure 4. (A) Oxidative modifications found in Ox_2 samples for each residue after 30 min and 2 h of oxidation. The algorisms in the table represent the total number of modified residues for each type of oxidative modification. Graphically shown are the total number of modified residues (regardless of the type of oxidative modification) for the two time-points presented. (B) BSA polypeptide sequence with corresponding domains and disulfide bridges (adapted from Brown[42]). Oxidized residues (balls) and protein sequence coverage (underlined) are shown for each time-point in the oxidative damage for Ox_2 samples. In the case of the time-points of 1 and 2 h, only the newly oxidized residues and the newly identified sequences are highlighted.30 min: —1 h: —2 h: —

In our results, the protein sequence coverage increases with higher oxidant concentrations and with the time-course of the oxidation reaction. With Ox_2 conditions, after 30 min and 1 h of oxidative damage, sequence coverage remained in the range between 47–50%, similarly to control samples, whereas, after 2 h, sequence coverage rose to 68.8%. In the Ox_10 samples, sequence coverage started at 62% after 30 min of oxidation, rose to 67% after 1 h and after 2 h it was as high as 75%.

The main affected regions of albumin, under the milder oxidative conditions (Ox_2), are in domains I, II-c and III. Additionally, the oxidative damage affects these regions at different points in the time-course evaluation (see Fig. 4(B)).

After 30 min, we identify 21 modified residues, mainly from domains I-b, I-c, II-c and domain III. Figure 5 shows the percentage of modified residues in each domain (considering the sequence coverage for each domain). The main affected residues in domain I are cysteine, tyrosine and proline. Oxidation also leads to modification of arginine and tyrosine residues from domain II-c and residues such as lysine, proline and histidine from domain III. We detect also, after 30 min, oxidation of all methionine and tryptophan residues (which have a low abundance in BSA) in domain I and 50% of the methionines in domain III. At this time-point (30 min) the free Cys-58 and the Cys-115 are also oxidized. Because Cys-115 is a cysteine residue involved in a disulfide bridge, at this time-point the disulfide bridge between Cys-99 and Cys-115 is, most likely, absent.

After 1 h of oxidation, we identify 20 modified residues. Nine of these residues were not observed at 30 min of oxidation. These newly modified residues appear mostly in domains II-c and III-a, with oxidized cysteine, tyrosine, arginine and phenylalanine residues (Figs. 4(B) and 5). The further oxidation of domain III-a can be the result of oxidation of two other cysteines involved in disulfide

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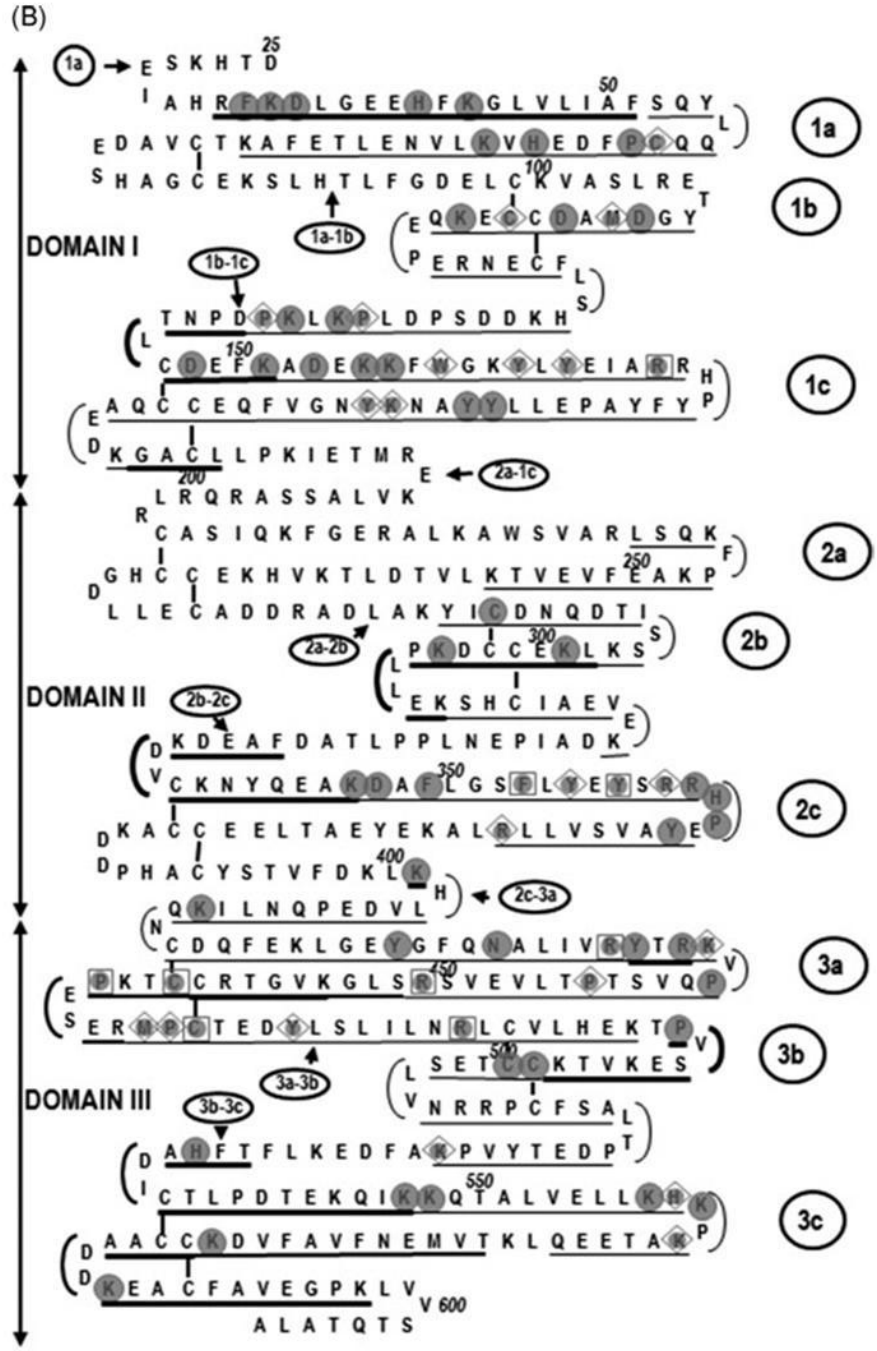

Figure 4. (Continued).

bridges, Cys-461 and Cys-471. This would help to spread the radical damage because of the disruption of the disulfide bridge between Cys-415 and Cys-461, and of the disulfide bridge between Cys-460 and Cys-471.

A higher number of identifications of oxidatively modified residues occurs after 2 h of oxidative damage, with 64 oxidized amino acids, of which 47 were not observed previously. These new residues belong to domain I, domains II-b and II-c, and domain III. The results suggest that between the period of 1 and 2 h of oxidation, albumin is no longer able to tolerate the oxidative damage and the consequent conformational changes provide an increased accessibility for the oxidant species. In the sample oxidized for 2 h, we identify three other oxidatively modified cysteines involved in disulfide bridges (Cys-288 from domain II-b and Cys-499 and Cys-500 from domain III-a). In result, the damage expanded faster throughout the protein sequence in domains II and III.

Under the more aggressive oxidative conditions (Ox_10) we identify a higher number of oxidatively modified residues. After just 30 min, as many as 50 residues were oxidatively modified (Supplementary Table 1, see Supporting Information). The main affected domains were domains I and III, in concurrence with the milder oxidative conditions, but also domains II-b and II-c. These results resemble the results from the Ox_2 samples after 2 h of oxidation, where the percentage of modified residues for each domain is similar, which is in concordance with the results obtained in

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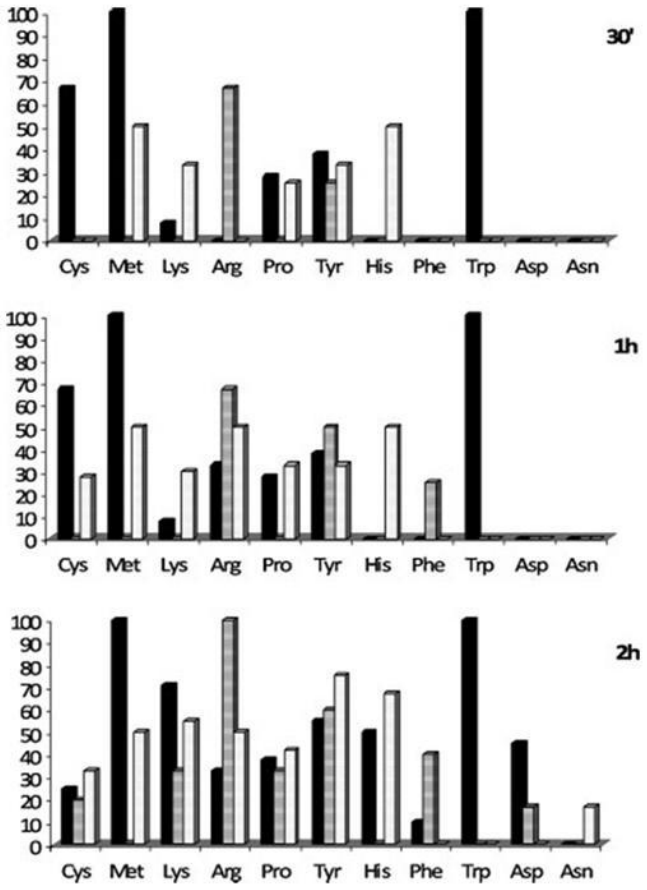

Figure 5. Percentage of modified residues per domain in Ox_2 samples, taking into account the coverage of each domain with oxidation. Black bars – domain I; striped bars – domain II; dotted bars – domain III.

the protein carbonylation experiments. We can notice affected residues such as cysteine, lysine, arginine, proline, tyrosine and aspartic acid in all the BSA domains and residues such as histidine, methionine, phenylalanine and asparagine in domains I and III. Again, besides the free Cys-58, at this time-point, we identify six other cysteine residues involved in disulfide bridges that are oxidized. We can find these disulfide bridges in domain II-b for oxidized Cys-312, in domain III-a for Cys-461 and Cys-471, and in domain III-b for Cys-484, Cys-499 and Cys-500. In this case, results suggest that at this time-point (30 min) the conformational changes are as severe as those shown for the Ox_2 samples after 2 h of oxidative damage.

Subsequently, after 1 h of oxidation with Ox_10 conditions, the number of newly identified oxidized residues is 32. These new residues are essentially cysteines, lysines, prolines, tyrosines and histidines from domains I-c, II, III-a and III-c. These findings are the consequence of further cysteine bridge disruption (oxidation of Cys-268, Cys-269 and Cys-276 in domain II-a) and consequent damage propagation across the protein.

After 2 h of oxidation, 71 residues were oxidatively modified, of which 20 were new residues. These residues are essentially located in domains I-c, II-a and II-c.

Under both oxidation conditions, Ox_2 and Ox_10, there is the case of a few residues that were found to be oxidatively modified at a time-point of the oxidative damage (e.g., 30 min) and not found in the following time-point (e.g., 1 h). We suggest the change in the relative abundance of oxidatively modified peptides from one time-point to another (mainly in result of the formation of new oxidative damage sites) means that it is not possible to identify all of the modifications (new and pre-existent). This in turn does not necessarily mean that these modifications are not present or occurring in the following time-points of the oxidative damage.

Although we were not able to detect fragmentation products in the gel electrophoresis analysis, fragmentation of the protein is also an event that takes place. When comparing the list of tryptic peptides from oxidized and nonoxidized albumin samples, we found tryptic peptides that are observed only in oxidized samples (Table 2). Though

Table 2. List of the tryptic peptides identified only in oxidized albumin samples. Peptide sequences (in bold) with the corresponding position and domain are shown for each sample. The associated errors presented values below 20ppm. √ means that the corresponding peptide was identified in the sample. Underlined residues show the positions that do not stand for a typical trypsin digest target

			Ox_2			Ox_10		
Sequence	Position	Domain	30 min	1 h	2 h	30 min	1 h	2 h
Q.**YLQQCPFDEHVK**.L	54–65	I-a				√	√	
Y.**LQQCPFDEHVK**.L	55–65			√		√	√	√
R.**RHPYFYAPE**.L	168–176	I-c		√	√	√		√
R.**HPYFYAPELLY**.Y	169–179				√		√	√
F.**YAPELLYYANK**.Y	173–183				√			
Y.**APELLYYANK**.Y	174–183					√		
E.**LLYYANK**.Y	177–183				√			
F.**LGSFLYEYSR**.R	350–359	II-c			√	√	√	√
L.**GSFLYEYSR**.R	351–359					√	√	√
S.**FLYEYSR**.R	353–359				√			
R.**RHPEYAVSVL**.L	360–369		√		√	√	√	
K.**LGEYGFQNAL**.I	421–430	III-a			√		√	
L.**GEYGFQNALIVR**.Y	422–433							√
V.**ELLKHKPKAT**.E	554–563	III-c				√		

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oxidation can be responsible for exposing new sites for tryptic digestion and thus the higher number of peptides identified in oxidized samples, these peptides do not show a typical tryptic cleavage site. It is also possible to notice that some of the identified peptides differ in one amino acid residue. These sequences can point to possible sites of protein backbone cleavage occurring near tyrosine, leucine and glycine residues. These peptides are all located in long loop regions positioned in domains I-a, I-c, II-c, III-a and III-c. For the Ox_10 samples, we identify almost all these peptides at the first time-point (30 min). However, for the milder oxidative conditions (Ox_2) we identify the peptides positioned in different domains at different time-points. After 30 min, we identified the peptide RHPEYAVSVL from domain II-c and, as already mentioned, this region is at this time-point affected with the two oxidized arginines and one oxidized tyrosine. In addition, newly oxidized residues appear in this region after 1 h of oxidation. As the oxidation reaction develops, we detect more peptides in other regions like domains I-a, I-c and III-a. Again, this data agrees with the identification of newly oxidized residues in the time-course of the oxidation process.

In conclusion, it is clear the result of the oxidative damage to albumin does not occur in a linear or progressive manner. This is recognizable when correlating data from the carbonyl assay, gel electrophoresis and mass spectrometry approaches. The increase in the carbonyl group formation and the number of newly modified residues identified between different time-points is nonlinear. Moreover, there is a significant decrease in the original BSA band between 1 and 2 h of oxidation. Altogether it suggests that the oxidative damage to albumin may proceed through a step-ladder mechanism.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

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3. *Insulin non-enzymatic glycation*

Mass Spectrometry Characterization of the Glycation Sites of Bovine Insulin by Tandem Mass Spectrometry

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Bovine insulin was glycated under hyperglycemic reducing conditions and in nonreducing conditions. Purification through HPLC allowed isolating glycated forms of insulin and a novel triglycated form (6224.5 Da) was purified. Endoproteinase Glu-C digestion combined with mass spectrometry (MALDI-TOF/TOF) allowed determining the exact location of the glycation sites in each of the isolated glycated insulins. For the first time, a triglycated form of insulin was isolated and characterized accordingly to its glycation sites. These glucose binding sites were identified as the N-terminals of both chains (Gly1 and Phe1) and residue Lys29 of B-chain. Moreover, in diglycated insulin we found the coexistence of one specie glycated at the N-terminals of both chains (Gly1 and Phe1) and another specie containing the two glucitol adducts in B-chain (Phe1 and Lys29). Also, in monoglycated insulin generated in reducing and nonreducing conditions, one specie glycated at Phe1 and another specie glycated at Lys29, both B-chain residues coexist. (J Am Soc Mass Spectrom 2009, 20, 1319–1326) © 2009 American Society for Mass Spectrometry

Over the last years, particular attention has been given to the role of nonenzymatic glycation in diabetic complications development, including neuropathy, nephropathy, retinopathy, and cardiovascular disease [1]. Under conditions of hyperglycemia, glycation compromises several functional proteins, such as hemoglobin [2, 3], glucose-6-phosphate dehydrogenase [4, 5], aldehyde reductase [6], glutathione reductase [7, 8], glutathione peroxidase [8, 9], and Cu-Zn superoxide dismutase [10, 11]. In this context, insulin glycation is of particular interest. The structurally modified peptide exhibits decreased ability to regulate plasma glucose homeostasis and is associated with reduced hepatic clearance, decreased adipose tissue lipogenesis, and a decreased glucose uptake and oxidation in isolated diaphragm and abdominal muscle in vitro [12–15]. Even though the short half-life of insulin (5–10 min), experiments performed to date indicate that a substantial proportion of insulin (and proinsulin) is glycated in the pancreatic cells during the stages of insulin synthesis and storage [16]. Glycated insulin has been measured in the pancreas of various animal models of type 2 diabetes [17], and in both isolated islets and clonal β-cells exposed to elevated glucose concentrations in tissue culture [18, 19]. The significant compromised biological activity of glycated insulin raises the possibility of its contribution to insulin resistance and glucose intolerance of type 2 diabetes.

Earlier studies indicated that during glycation process, each insulin molecule can bind to ~three to eight molecules of glucose [14, 20]. More recently, independent reports generalized the view that there are three possible free amino groups in the insulin molecule, human or bovine, available for in vitro glucose binding [15, 21–23]. Nowadays, through ESI-MS/MS and MALDI-MS approaches, it is established the N-terminal Phe1 residue of the B-chain is the only glycation site of human [23] and bovine [21] insulin. Moreover, O'Harte and coworkers determined, in a novel diglycated insulin (under hyperglycemic reducing conditions), the N-terminals Gly1 of A-chain and Phe1 of B-chain as the sites of glycation for human insulin [15], after Glu-C enzymatic digestion and identification of digested fragments by plasma desorption mass spectrometry (PDMS). Since human and bovine insulin only differ in three residues (A chain: residues 8 and 10; B chain: residue 30), it is expected that glycation of bovine insulin should occur in similar positions.

The present study structurally characterizes three forms of glycated bovine insulin when glycated under reducing conditions. Simultaneously, the characterization of glycated insulin produced under pseudophysiological conditions was also done. Our approach included HPLC purification of mono-, di-, and triglycated insulin forms, followed by enzymatic digestion and mass spectrometry (MALDI-TOF/TOF) for the unambiguous assignment of the glycation sites.

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Materials and Methods

Chemicals and Reagents

Bovine insulin (I5500) and TFA were acquired from Sigma (St. Louis, MO). The MALDI matrices α-cyano-4-hydroxy cinnamic acid (CHCA) and sinapinic acid were supplied by Merck (Darmstadt, Germany). The organic solvents such as toluene and acetonitrile (ACN) were from Riedel-deHaën (Buchs, Switzerland) and Labscan (Dublin, Ireland), respectively.

All chemicals were of analytical grade and milli-Q water (Millipore, Bedford, MA) was used throughout all experiments.

Insulin In Vitro Glycation

In vitro glycation was carried out by incubating insulin with D-glucose in a protocol adapted from O'Harte et al. where reducing [23] and nonreducing conditions were used. In nonreducing conditions, insulin (200 μg in HCl 2 mM) was incubated with D-glucose 220 mM in phosphate buffer 10 mM (pH 7.4) at 37 °C for 30 d (toluene 5 mM was added as a bacteriostatic). In reducing conditions and according to the authors, insulin (100 μg in HCl 2 mM) was incubated for 24 h at 37 °C with D-glucose 220 mM (prepared in phosphate buffer 10 mM, pH 7.4) together with a 1000-fold molar excess of the reducing agent $NaBH_3CN$ (stock in ice cold NaOH 10 mM) in phosphate buffer 10 mM (pH 7.4). In both cases, after incubation, reaction was stopped by addition of acetic acid 0.5 M, and the reaction mixture was dialyzed overnight against water (cut-off 1000 Da). Glycated and native forms were separated on a C-8 analytical column (Acclaim, 4.6 × 250 mm, 5 μm particle size; Dionex, Sunnyvale, CA) at a flow rate of 0.8 mL/min. The program was as follows: linear gradient of 0% to 35% ACN over 10 min, followed by 35% to 56% ACN over 20 min, and 56% to 70% ACN over 5 min. Peaks were hand-collected, pooled from several runs, and concentrated on a Speed-vac. Re-injection of each glycated form using the same separation program allowed to improve the purification of each glycated insulin form. This purification was monitored through MALDI-MS.

Enzymatic Digestion (Endoproteinase Glu-C)

For in-solution digestion of glycated and native insulin, samples were initially reconstituted in HCl 2 mM. Quantification was performed using the Quant-iT Protein Assay kit (Invitrogen, Paisley, UK) combined with the Qubit fluorometer. An aliquot was taken and diluted for enzymatic digestion with phosphate buffer 10 mM pH 7.8; pH is determinant since the specificity of endoproteinase Glu-C for glutamic acid depends on this parameter. Endoproteinase Glu-C (Calbiochem, Merck, Darmstadt, Germany) solution was added in a final ratio of substrate: enzyme of 20:1 (wt/wt). Duplicate samples were incubated overnight at 25 °C. After digestion, samples were dried under vacuum in a Speed-vac and, unless taken for analysis, stored at −80 °C.

Reduction of Disulphide Bridges of Insulin

The disulphide bridges of monoglycated, diglycated, triglycated, and native insulin were reduced using DTT 10 mM in phosphate buffer pH 8 for 1 h at 55 °C. An aliquot of each sample (10 μL) was taken and acidified with TFA 1% (1 μL). These aliquots were cleaned up using ZipTip C18 (Millipore, Bedford, MA) before mass spectrometry analysis.

Mass Spectrometry

Glu-C digests of glycated and native insulin were reconstituted in TFA 0.1%. An aliquot of each sample was mixed (1:1) with α-CHCA matrix solution (5 mg/mL in ACN 50%/TFA 0.1%) and applied onto 384-well MALDI plates. Instrument was calibrated by adding insulin (12.5 ng; 0.5 μL) to each calibration spot. Peptide mass spectra were obtained with a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer; Applied Biosystems, Foster City, CA) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 7000 Da. Manual interpretation of tandem mass spectra was performed through the Data Explorer software TM ver. 4.4 (Applied Biosystems).

Results and Discussion

Glycation of insulin was monitored by MALDI-MS through the increase in the molecular weight of insulin as a result of glucose adducts formation with the hormone. Figure 1 shows the typical mass spectra of native (a) and glycated insulin (b and c). In Figure 1b, we can observe a typical spectrum of glycated insulin where glycation of the hormone was performed by incubation with 220 mM glucose for 30 d at 37 °C in the absence of reducing conditions. The major peak at *m/z* 5730.66 corresponds to native bovine insulin (theoretical *m/z* 5730.6) whereas the second peak at *m/z* 5892.72 with a mass shift of 162 Da corresponds to a monoglycated form of insulin. A third minor peak at *m/z* 6054.71 is also observable and should correspond to a diglycated form with a mass shift of 324 Da. Figure 1c, where insulin glycation was performed by incubation with 220 mM glucose for 30 h at 37 °C in reducing conditions ($NaBH_3CN$), shows a different mass profile, especially in terms of relative abundance of each specie. There are three additional peaks in the spectrum besides the peak corresponding to native insulin. The major peak, at *m/z* of 5894.72, is the protonated monoglycated form with a mass shift of 164 Da (glucitol adduct). The two other peaks at *m/z* 6058.89 and 6222.82 correspond to the protonated diglycated and triglycated forms, respectively. With the mono-, di-, and triglycated forms, several additional peaks are observed. These peaks are BH3-adducts (Δm = 14 Da), resulting from the in vitro

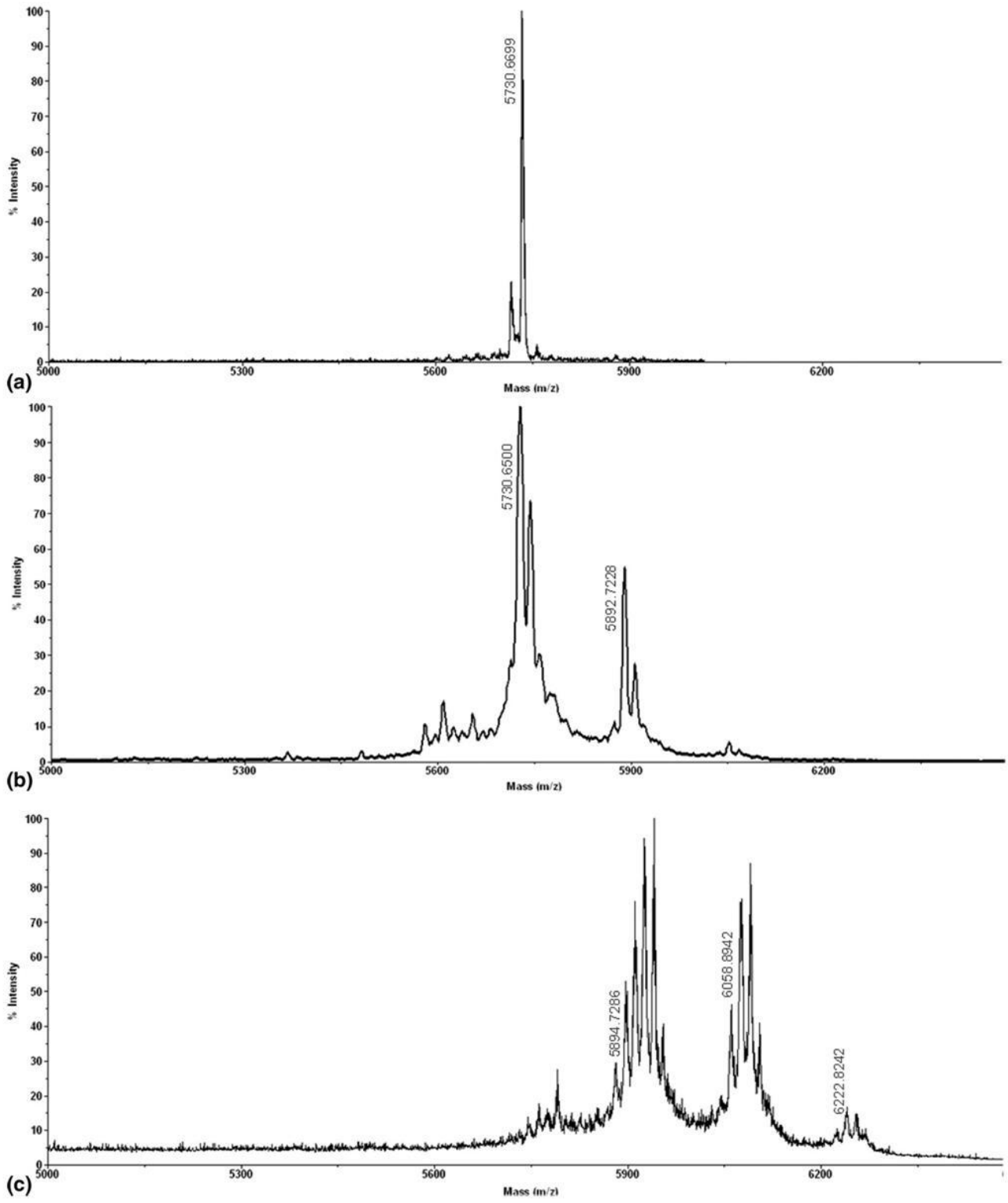

Figure 1. Typical MALDI-MS spectra of native insulin and glycated insulin in both reducing and nonreducing conditions. (**a**) Native insulin, (**b**) glycated insulin under nonreducing conditions, (**c**) glycated insulin under reducing conditions.

glycation procedure where an excess of this reducing agent is used.

After separating the different glycated forms of insulin by HPLC-C8, purified samples were reconstituted in HCl 2 mM and quantified with Quant-iT Protein Assay kit. In the absence of reducing conditions, 32% of the purified monoglycated form was obtained. In reducing conditions 45% of monoglycated, 25% of di-

glycated, and 6% of triglycated forms of insulin were obtained.

To identify the glycation sites, each sample was enzymatically digested with endoproteinase Glu-C as described in the experimental section. Cleavage of insulin at glutamic acid residues without disulfide bridges disruption theoretically produces nine fragments (Table 1). Digested samples of glycated and native insulin were analyzed by MALDI-MS, and Figure 2 shows representative spectrum of Glu-C digests for all studied samples. Highlighted peaks that are present solely in glycated samples were chosen for analysis through tandem mass spectrometry (MALDI-MS/MS). Table 1 shows the identified peaks in all samples. Figure 3 shows the two MS/MS spectra of the peptides at *m/z* 1248.57 (Figure 3a) and 1644.68 (Figure 3b), identified as fragments B1-13 and B22-30 from glycated insulin (no reducing conditions) with one glucose adduct attached (see Table 1).

Analysis of the MS/MS spectrum of the modified peptide at *m/z* 1248.57 (Figure 3a) allows locating the glucose adduct in residue Lys29. In the MS/MS spectrum, it is possible to see the mass difference of 290.2 Da between the ion at *m/z* 1131.5 (a8) and the ion at *m/z* 841.3 (a7). This gap corresponds to the loss of the lysine residue with a glucose adduct. Analysis of the MS/MS spectrum of the precursor ion at 1644.68 (see Figure 3b) allows locating the glucose adduct in residue Phe1. These results agree with the literature [21, 22], which state the most likely sites of glycation within the B-chain are the Phe1 and Lys29 residues, as both contain free amino groups for linking glucose molecules. Nevertheless, the preferred glycation site of monoglycated bovine or human insulin is thought to be at Phe-1 of the

Table 1. Theoretical peptide fragments from endoproteinase Glu-C digestion of native insulin and experimental glycated fragments obtained from Glu-C digests of glycated insulin samples. Underlined residues correspond to cysteines involved in disulfide bridges

Position	Peptide sequence	Theoretical mass (Da)	Experimental mass (Da)	Match error (Da)
Native insulin				
A 1-4	GIVE	417.2324	—	—
A 5-17	QCCASVCSLYQLE	1446.6062	—	—
A 5-17	QCCASVCSLYQLE	1444.5906	1444.4225	−0.1681
A 18-21	NYCN	513.1762	—	—
B 1-13	FVNQHLCGSHLVE	1482.7158	1482.6351	−0.0807
B 14-21	ALYLVCGE	867.4280	867.4041	−0.0239
B 22-30	RGFFYTPKA	1086.5731	1086.5048	−0.0683
A 5-17/B 1-13	QCCASVCSLYQLE/FVNQHLCGSHLVE	2924.283	—	—
A 18-21/B 14-21	NYCN/ALYLVCGE	1377.5808	1377.4717	−0.1091
Glycated insulin				
Monoglycated insulin (nonreducing conditions)				
B 14-21	ALYLVCGE	867.4280	867.4378	0.0098
B 22-30	RGFFYTPKA	1086.5731	1086.5291	−0.0440
B 22-30	RGFFYTPKA	1248.6259	1248.5710	−0.0549
A 18-21/B 14-21	NYCN/ALYLVCGE	1377.5808	1377.5029	−0.0779
A 5-17	QCCASVCSLYQLE	1444.5906	1444.5505	−0.0401
B 1-13	FVNQHLCGSHLVE	1482.7158	1482.6345	−0.0813
B 1-13	FVNQHLCGSHLVE	1644.7686	1644.6820	−0.0866
Glycated insulin (reducing conditions)				
Monoglycated				
B 22-30	RGFFYTPKA	1086.5731	1086.4946	−0.0785
B 22-30	RGFFYTPKA	1250.6415	1250.5546	−0.0869
A 18-21/B 14-21	NYCN/ALYLVCGE	1377.5808	1377.4662	−0.1146
A 5-17	QCCASVCSLYQLE	1444.5906	1444.5294	−0.0612
B 1-13	FVNQHLCGSHLVE	1646.7842	1646.6748	−0.1094
Diglycated				
B 22-30	RGFFYTPKA	1086.5731	1086.4970	−0.0761
B 22-30	RGFFYTPKA	1250.6415	1250.5597	−0.0818
A 18-21/B 14-21	NYCN/ALYLVCGE	1377.5808	1377.4740	−0.1068
A 5-17	QCCASVCSLYQLE	1444.5906	1444.5748	−0.0158
B 1-13	FVNQHLCGSHLVE	1646.7842	1646.7093	−0.0749
A 1-21	GIVEQCCASVCSLYQLENYCN	2501.0345	2501.0765	0.0420
Triglycated				
B 22-30	RGFFYTPKA	1086.5731	1086.4949	−0.0782
B 22-30	RGFFYTPKA	1250.6415	1250.5478	−0.0937
A 18-21/B 14-21	NYCN/ALYLVCGE	1377.5808	1377.4398	−0.1410
A 5-17	QCCASVCSLYQLE	1444.5906	1444.5100	−0.0806
B 1-13	FVNQHLCGSHLVE	1646.7842	1646.6378	−0.1464
A 1-21	GIVEQCCASVCSLYQLENYCN	2501.0345	2501.1065	0.0720

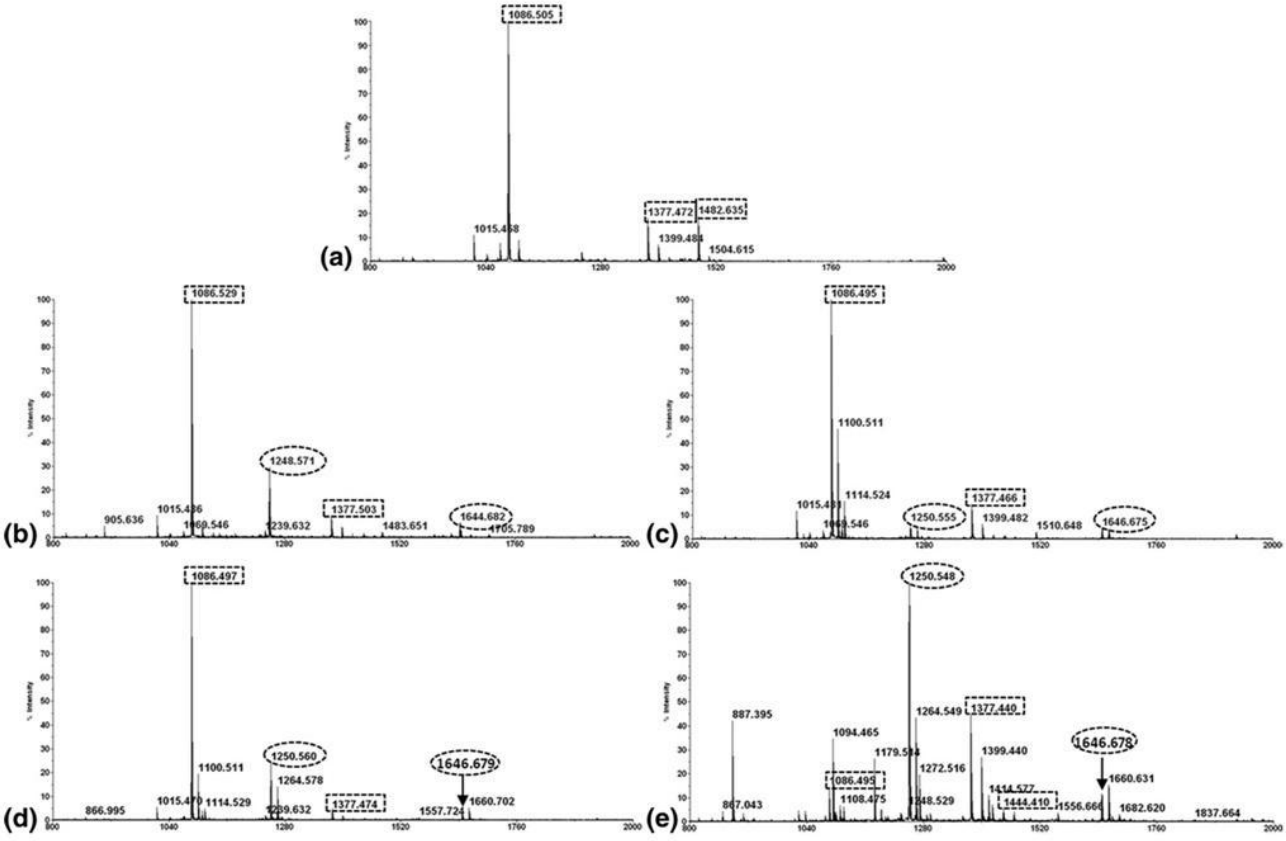

Figure 2. MALDI-MS spectra of Glu-C digests of native insulin and isolated forms of glycated insulin for both glycation procedures. The *m/z* values of fragments matching theoretical peptide fragments shown in Table 1 are highlighted with rectangles. Peptide fragments highlighted with circles in glycated samples are unique to these samples and correspond to glycated fragments. (**a**) Native insulin, (**b**) monoglycated insulin under nonreducing conditions, (**c**) monoglycated insulin under reducing conditions, (**d**) diglycated insulin (reducing conditions), (**e**) triglycated insulin (reducing conditions).

B-chain [23]. This is most probably due to the high reactivity as this residue is situated at the N-terminal of the peptide chain. Herein, we show that monoglycated insulin produced in the absence of reducing conditions comprises the coexistence of two glycated insulin species with two different glycation sites, Phe1 or Lys29.

Figure 4 shows three representative examples of MS/MS spectra of glycated fragments belonging to glycated insulin produced under reducing conditions. The two glycated fragments that result from Glu-C digestion are the peptides at *m/z* 1250.56 and *m/z* 1646.67, and were found in all of three forms of glycated insulin (see Figure 2). These peptides were fragments B1-13 and B22-30 with identical glycation sites, Phe1 and Lys29, respectively, as reported for the monoglycated insulin formed in the absence of reducing conditions. In this case, because of the excess of reducing agent ($NaBH_3CN$), the mass shift found in the MS/MS spectra corresponds to the attachment of a glucitol adduct (164 Da). Again, the coexistence of two glycated forms occurs in monoglycated insulin produced under reducing conditions.

With the approach described so far, it was not possible to identify any glycated fragments belonging to the A-chain in monoglycated, diglycated and triglycated forms of insulin. To induce the separation of the two chains (A and B) of the insulin samples, the disulphide bridges were reduced with DTT using an aliquot of undigested samples, as described in the Experimental section. These reduced samples were submitted to MALDI-MS and MALDI-MS/MS analysis. Figure 4c shows the representative MS/MS spectrum of the A-chain containing a single glucitol adduct, with an *m/z* 2501, which was found only in diglycated and triglycated forms of insulin. The identification of the complete *b* series allowed the identification of Gly1 as the glycation site in the A-chain. This result allowed determining the three sites of glycation in triglycated insulin and also pointed out the presence of two isoforms in diglycated insulin, similar to the monoglycated forms of insulin. Therefore, in diglycated insulin, there is the coexistence of one specie glycated at the N-terminals of both chains (Gly1 and Phe1) and another specie containing the two glucitol adducts in chain B (Phe1 and Lys29).

Furthermore, we calculated the relative abundance (RA), in percentage, of glycated peptides for monoglycated and diglycated samples. As normalizing reference

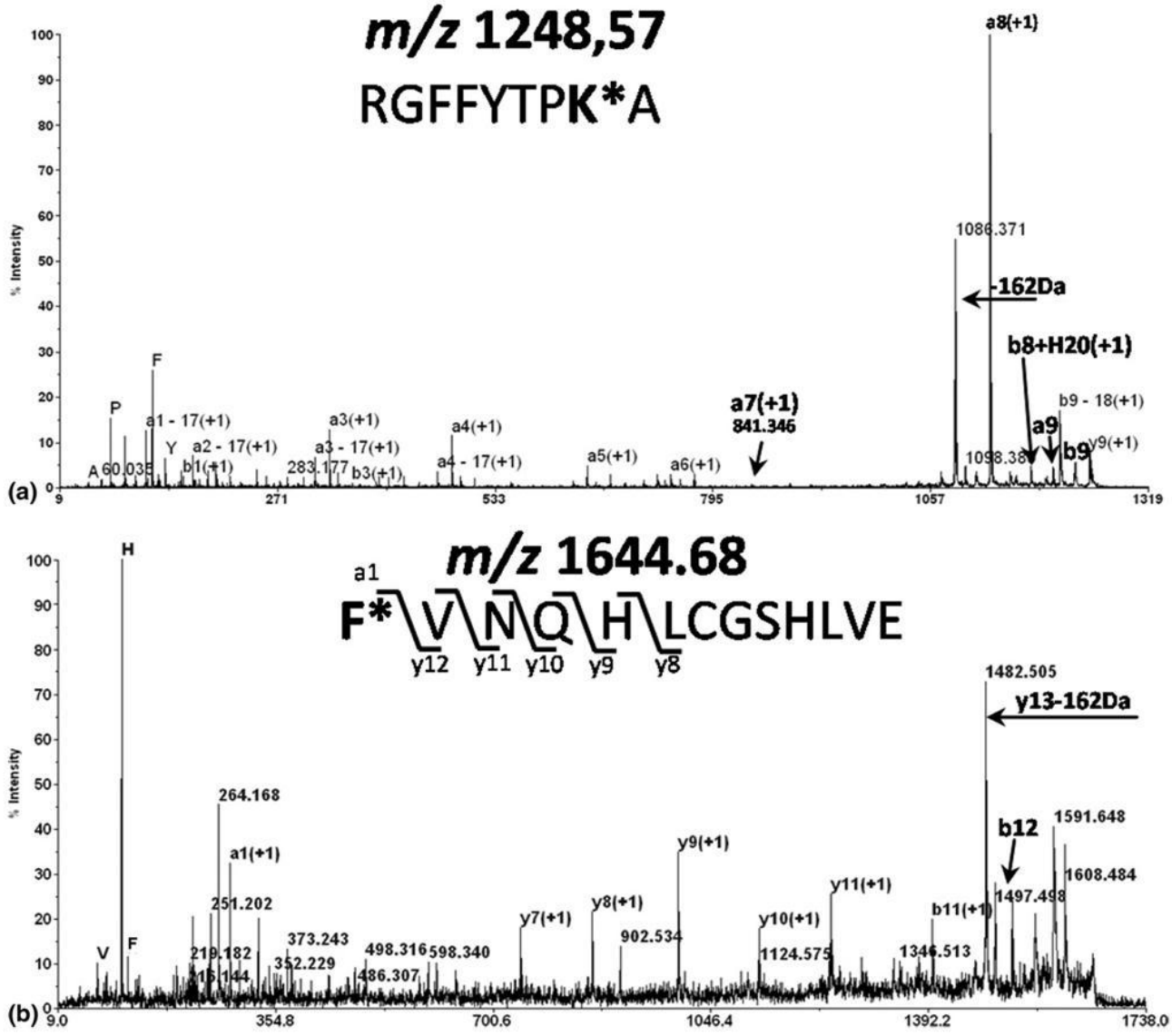

Figure 3. MS/MS spectra of the modified peptide at *m/z* 1248.57 (**a**), corresponding to the sequence between B 22–30 with a glucose adduct located at Lys29, and the modified peptide at *m/z* 1644.68 (**b**), corresponding to the sequence between B 1–13 glycated at Phe1. These glycation sites were found in monoglycated insulin produced under nonreducing conditions.

peak, we used the peak at *m/z* 1086 (chain B residues 22–30) since it is the most abundant peak in GluC-digested samples. This analysis indicate that the monoglycated insulin of Figure 1b is preferably glycated at Lys29 (Lys29 RA = 30.7% ± 0.8%; Phe-1 RA = 5.7% ± 0.5%), while in the monoglycated insulin of Figure 1c, the two possible glycation sites present a similar abundance (Lys29 RA = 6.0% ± 0.1%; Phe1 RA = 5.1% ± 1.3%). In the case of diglycated insulin from Figure 1c, although also glycated at Gly1 of chain A, data indicate it appears to be preferably glycated at Lys29 (Lys29 RA = 23.5% ± 0.3%; Phe1 RA = 4.6% ± 0.7%). Therefore, this diglycated form appears to coexist in two isoforms, one glycated at Phe1 and Lys29 of chain B and the other glycated at Lys29 (chain B) and Gly1 (chain A).

This is the first time that a triglycated form of insulin was isolated and characterized according to its glycation sites. In addition, we were also able to identify in diglycated insulin, the simultaneous presence of two isoforms where three sites of glycation are possible, rather than solely the N-terminals of both chains (Gly1 and Phe1), as reported for human insulin [15]. Therefore, the importance of Lys29 as a possible site of glycation occurring in vivo should not be ignored, as also shown by the results corresponding to the monoglycated insulin produced in nonreducing conditions. To date, the impairment of in vivo and in vitro actions of insulin when glycated has only been studied with insulin glycated under reducing conditions [12, 13, 15], and therefore only considered to be glycated at the N-terminal Phe1 of the B-chain [13] or

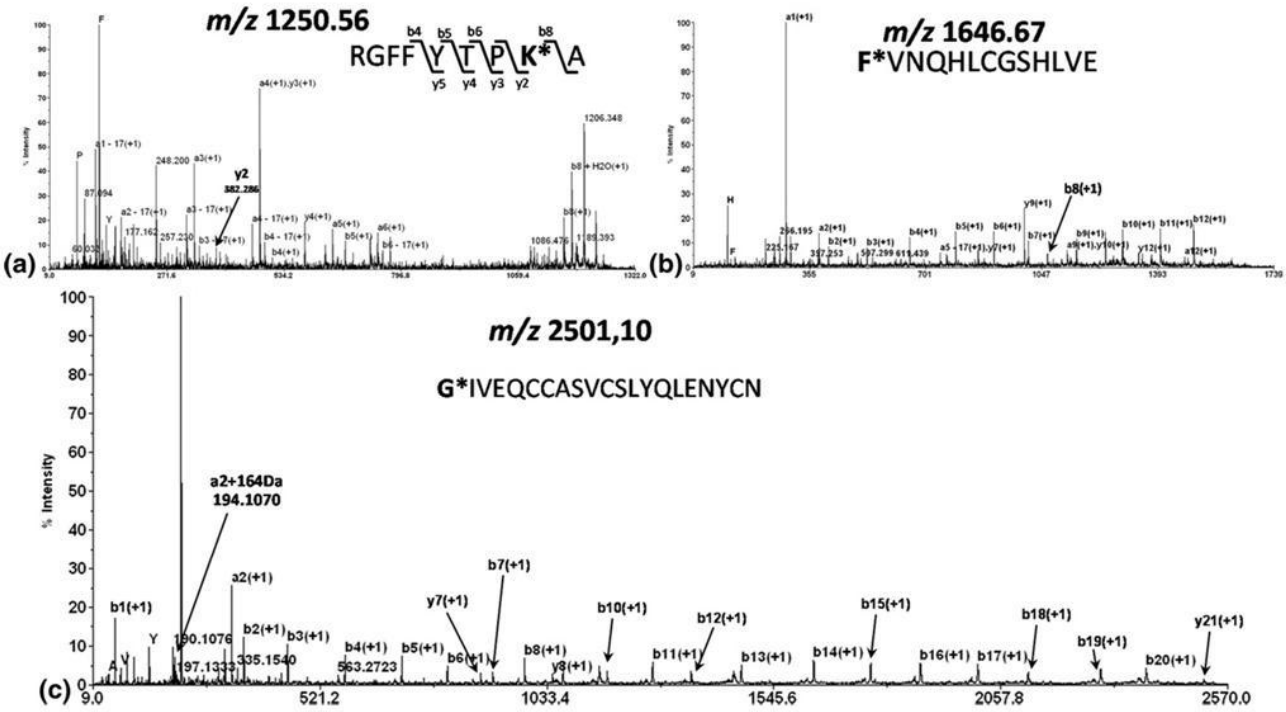

Figure 4. MS/MS spectrum of the modified peptide at *m/z* 1250.56 (**a**), corresponding to the sequence between B 22–30 with a glucose adduct located at Lys29. (**b**) Shows the MS/MS spectrum of the modified peptide at *m/z* 1646.67, which corresponds to the sequence between B 1–13 glycated at Phe1. (**c**) shows an MS/MS spectrum representative of the A-chain with a single glucitol adduct at *m/z* 2501.10, where the glycation site is located at Gly1. These glycation sites were found in glycated insulin produced under reducing conditions.

glycated at the N-terminals of both chains (Gly1 and Phe1) [15]. It would be of interest to develop further work with glycated insulin at Lys29, since insulin glycation in vivo is associated with characteristic complications of type 2 diabetes such as glucose intolerance and insulin resistance. Elucidating the importance of the structural alterations, which occur in consequence of glycation, in the modulation of insulin action should continue to be of great interest.

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4. Interrelationship between glycation and oxidation

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ORIGINAL PAPER

Oxidative modifications in glycated insulin

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Abstract At the present, the term "glycoxidation" is recognized as the synergistic interaction between glycation and oxidative processes which, with the help of redox-active metals, consequently leads to the production of deleterious tissue modifications. The association between glycation and oxidation events is considered one of the major factors in the accumulation of non-functional damaged proteins, enhancing the oxidative damage at the cellular level. Because of the central role of insulin in the biology of diabetes, we investigated the site-specific oxidation of native and glycated insulin (mono, di, and tri-glycated forms), through metal-catalyzed oxidation, with a combination of liquid chromatography and mass spectrometry. With this approach we were able to identify the residues that were mainly oxidized, and peptide sequences resulting from oxidative cleavage of insulin. Tyrosine, phenylalanine, and cysteine were the main affected residues. Time-course analysis (0–48 h) of the oxidative damage enabled to detect more pronounced and earlier oxidative modifications in the case of glycated insulin. We also observed more severe oxidative damage as the number of glycation sites increased in insulin. These oxidative modifications included other oxidized residues, namely proline, histidine, valine, leucine, and glycine, which were shown to be carbonylated. In addition, we identified new sites of peptide cleavage with the formation of new fragments, derived mainly from chain B, which were both glycated and oxidatively modified. Peptide fragmentation occurred mainly between the residues phenylalanine, glycine, leucine, and tyrosine. Moreover, for diglycated and triglycated forms we observed further oxidative cleavage occurring in both chains, with oxidation and fragmentation of residues occurring near cysteine bridges, especially in chain A.

Keywords Insulin · Glycation · Oxidative damage · Oxidative stress · Mass spectrometry

Introduction

It is widely acknowledged that oxidative modification of proteins by reactive oxygen species (ROS), or other reactive substances (RS), is implicated in normal aging, and in the etiology or progression of a number of physiological disorders and diseases [1–3]. These reactive species may be generated by a large number of physiological and non-physiological processes (reviewed in Ref. [3]) and, nowadays, it is well established that these species are of great importance in the modification of proteins [4–7], lipids [8, 9], and nucleic acids [9, 10]. Among these biomolecules, proteins are the principal target for damage caused by radicals and other oxidants. According to published rate constants and cellular abundance of proteins, it has been estimated that proteins can scavenge 50% to 75% of reactive radicals [11].

Most current knowledge about the modification of proteins by ROS was obtained from in-vitro studies [12–24] of the effects of radiation, metal catalyzed oxidation, and direct reaction with oxygen radicals. Those reports enabled establishment of the principles and concepts which are crucial for improving the understanding of the mechanisms of protein and amino acid oxidative modification. Results of these studies showed that oxidation of proteins by ROS leads mainly to

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oxidation of amino acid residue side chains, fragmentation of the polypeptide chain, and formation of covalent protein–protein cross-linked derivatives (reviewed in Ref. [25]). The known products result from hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and conversion of some amino acid residues to carbonyl derivatives [25, 26].

Recently, Olivares-Corichi and co-workers [27] reported an in-vitro study in which the effects of ROS on insulin were evaluated. The authors used the Fenton reaction for hydroxyl radical generation and focused on the chemical changes of the structure of insulin monitored by spectrophotometric methods and radioimmunoassay. Results pointed to the formation of phenylalanine hydroxyl derivatives, formation of peroxidation products on amino acids side branches, increased concentration of free carbonyl groups, and reduced insulin antigen–antibody reactivity. Recently, the oxidative modification of albumin was evaluated using a metal-catalyzed oxidation (MCO) in-vitro system [28]. The use of a mass spectrometry (MS) based approach, combined with off-line nano liquid chromatography, enabled the study of structural changes and the time-course evaluation of these changes on albumin during the oxidative damage. Taking advantage of the high potential of this MS-based approach, we analyzed the oxidative modifications of insulin when subjected to in-vitro oxidation.

Because insulin is extremely important in cellular glucose uptake, we also considered the relevance of this hormone in the worldwide increase of insulin resistance occurring in clinical settings such as diabetes [29]. Previous studies have shown that glycated insulin has impaired ability to regulate plasma glucose and stimulate glucose transport and metabolism [30, 31]. Also, it is accepted that increased oxidative stress is a factor in the development and progress of diabetes and its complications [32]. The term "glycoxidation" reflects this synergistic interaction between glycation and the oxidative processes which, in turn, are responsible for causing deleterious tissue modifications with the help of redox-active metals, these events being of high importance in a pathological condition such as diabetes. Because of the central role of insulin in the biology of diabetes, we further investigated the oxidative behavior of glycated insulin with the determination of site-specific oxidative damage of both native and glycated insulin. The glycated insulin samples employed in this study have previously been characterized by our group and data have been published elsewhere [33]. Previously, Cheng and Kawakishi [34], investigated site-specific oxidation of glycated bovine insulin mediated by copper ions and detected oxidative modification of two histidines in the B-chain by use of amino acid composition and sequence analyses. The authors also observed fragmentation at alanine, proline, valine, leucine, and isoleucine residues.

Experimental

Chemicals and reagents

Bovine insulin (I5,500) and TFA were acquired from Sigma (St Louis, MO, USA). The MALDI matrix, α-cyano-4-hydroxycinnamic acid (CHCA), was supplied by Merck (Darmstadt, Germany). Organic solvents such as toluene and acetonitrile (ACN) were from Riedel–deHaën (Buchs, Switzerland) and Labscan (Dublin, Eire), respectively.

All chemicals were of analytical grade and Milli-Q water was used throughout all experiments.

Insulin in vitro glycation

In-vitro glycation was carried out by incubating insulin with glucose under pseudo-physiological conditions and according to O'Harte et al. [35]. In the first case, insulin (200 μg in 2 mmol L^{-1} HCl) was incubated with 220 mmol L^{-1} D-glucose in 10 mmol L^{-1} PBS (pH 7.4) at 37°C for 30 days (NRed1g), with 5 mmol L^{-1} toluene added as a bacteriostatic. In the second case, insulin (100 μg in 2 mmol L^{-1} HCl) was incubated for 24 h at 37°C with 220 mmol L^{-1} D-glucose (prepared in 10 mmol L^{-1} PBS, pH 7.4) together with a 1000-fold molar excess of the reducing agent $NaBH_3CN$ (stock in ice cold 10 mmol L^{-1} NaOH) in 10 mmol L^{-1} PBS (pH 7.4) (Red1g, Red2g, and Red3g). After incubation, reaction was stopped by addition of 0.5 mol L^{-1} acetic acid and the reaction mixture was dialyzed overnight against water, using a membrane (Spectra, VWR) with cut-off 1000 Da. In both procedures, glycated and native forms were separated on a C_8 analytical column (Acclaim, 4.6 mm×250 mm, 5-μm particle size, Dionex) at a flow rate of 0.8 mL min^{-1}. The program was as follows: linear gradient of 0–35% ACN over 10 min, followed by 35–56% ACN over 20 min and 56–70% ACN over 5 min. Peaks were hand-collected, pooled from several runs, and concentrated on a Speed-vac. Re-injection of each fraction using the same separation program enabled improvement of the purity of each glycated form of insulin. This purification was monitored by use of MALDI-MS. These purified glycated forms of insulin were characterized and the corresponding data published in an independent report [33].

Insulin in vitro oxidation

All oxidation reactions were performed at 37°C and Fenton oxidant was chosen for metal-catalyzed oxidation.

Purified native and glycated forms of insulin were submitted to in-vitro oxidation, and a typical oxidation reaction mixture contained 25 μmol L^{-1} insulin, 50 μmol L^{-1} $FeCl_2$/EDTA, and 100 μmol L^{-1} H_2O_2, in 10 mmol L^{-1} PBS (pH 7.4). After incubation for different times (4, 24, and 48 h), the reactions were stopped with 1% TFA and, unless taken for further analysis, each sample was stored at −80 °C. In parallel, control reactions for both native and glycated forms of insulin were performed by incubating insulin alone in phosphate buffer and incubating insulin in the absence of H_2O_2 but maintaining the presence of $FeCl_2$/EDTA. All experiments were performed in duplicate and oxidative damage was monitored by use of MALDI-MS. After the indicated incubation time, an aliquot of each sample (5 μL) was mixed (1:1) with α-CHCA matrix solution (5 mg mL^{-1} in ACN 50%–TFA 0.1%) and applied to 384-well MALDI plates. The instrument was calibrated by adding insulin (12.5 ng; 0.5 μL) to each calibration spot. Peptide mass spectra were obtained with a MALDI-TOF–TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive-ion reflector mode. Spectra were obtained in the mass range between 800 and 7000 Da.

Enzymatic digestion, nano-RP-HPLC, and MALDI fraction collection

For in-solution digestion of control and oxidized insulin (native and glycated), samples were initially diluted with 10 mmol L^{-1} PBS, pH 7.8. Endoproteinase Glu-C (Calbiochem, Merck, Darmstadt, Germany) solution was added in a final ratio of substrate:enzyme of 20:1 (*w*/*w*). Digestion was performed in duplicate and samples incubated overnight at 25 °C. After digestion, samples were dried under vacuum in a Speed-vac and, unless taken for analysis, stored at −80 °C.

Glu-C digests of non-oxidized and oxidized insulin samples were subjected to separation by liquid chromatography using an Ultimate 3000 column (Lc Packings). Oxidized insulin samples (native and glycated) not submitted to in-solution digestion were also subjected to separation by liquid chromatography. In all cases, for each sample, 2 μg total protein was automatically injected on to a C_{18} trapping column (Zorbax 300SB-C18, 5-μm particle size, 5 mm×0.3 mm, Agilent Technologies). Samples were washed over the trapping column for 3 min with a linear gradient of 95% buffer A (water, TFA 0.1%) and 5% buffer B (ACN 80%, TFA 0.04%) at a flow rate of 30 μL min^{-1}. After 13 min, the flow was redirected to the analytical column and the sample eluted on to a 150 mm× 75 μm Zorbax 300SB capillary analytical C_{18} column (3.5 μm particle size, Agilent Technologies) at a flow rate of 0.3 μL min^{-1}. Tryptic peptides were separated by using a linear gradient of 5–15% buffer B over 10 min, 15–50% buffer B over 47 min, 50–90% buffer B over 2 min, followed by a 3-min hold at 90% buffer B. The peptides eluting from the monolithic capillary column were directly deposited on to 384-well MALDI plates, using a Probot (Lc Packings), together with simultaneous application of 170 nL α-CHCA matrix solution.

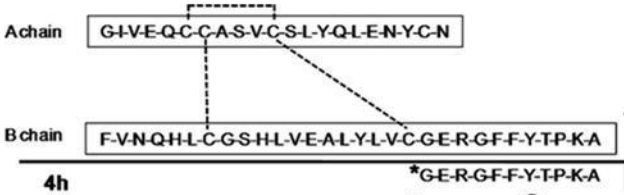
Mass spectrometry and database search

Peptide mass spectra were obtained with a MALDI-TOF–TOF mass spectrometer (4800 Proteomics Analyzer,

Table 1 List of oxidative modifications updated from the UNIMOD web site and used for database searching of data from tandem mass spectrometry (MS–MS) through the Protein Prospector Server

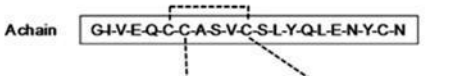
Modification	Description	Δ*m* (monoisotopic)
Amino (Tyr)	Tyrosine oxidation to 2-aminotyrosine	15.0108
Arg → GluSA	Arginine oxidation to glutamic semialdehyde	−43.0534
Cys → Oxoalanine	Cysteine oxidation to oxoalanine	−17.9928
His → Asn	Histidine oxidation to aspargine	−23.0159
His → Asp	Histidine oxidation to aspartic acid	−22.0319
Lys → Allysine	Lysine oxidation to aminoadipic semialdehyde	−1.0316
Lys → Aminoadipic acid	Lysine oxidation to α-aminoadipic acid	14.9632
Pro → Pyrrolidone	Proline oxidation to pyrrolidone	−27.9949
Pro → Pyrrolidinone	Proline oxidation to pyrrolidinone	−30.0105
Pro → Pyro-Glu	Proline oxidation to pyroglutamic acid	13.9792
Trioxidation (Cys)	Cysteine oxidation to cysteic acid	47.9847
Oxidation and dioxidation	Oxygen addition and hydroxylation (Lys, Arg, Cys, Tyr, His, Pro, Phe, Val, Leu, Gly, Asn)	15.9949
Carbamylation (Lys, Arg, Cys)	Isocyanate reaction with amino groups	43.0058

Applied Biosystems) in the positive-ion reflector mode. Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots. Tandem mass spectral (MS–MS) data from each sample spot were processed using the Protein Prospector Server v4.27.2 (University of California, San Francisco, USA), using the following settings: no enzyme/Glu-C, SwissProt accession number P01317, variable mods (oxidation and trioxidation) plus



Native insulin

		Observed mass	Match error PPM	Modification
4h	*G-E-R-G-F-F-Y-T-P-K-A	1272,6410	3	
	*G-E-R-G-F-F-(Y)-T-P-K-A	1288,6188	-10	Oxidation (Y)[26]
24h	*S-H-L-V-E-A-L*	768,4325	9	
	*G-E-R-G-F-(F)-Y-T-P-K-A	1288,6639	25	Oxidation (F)[25]
	E-R-G-F-(F)-Y-T-P-K-A	1231,6273	14	Oxidation (F)[25]
	E-R-G-F-F-Y-T-P-K-A	1215,6451	24	
	R-G-F-F-Y-T-P-K-A	1086,5977	23	
48h	*V-E-A-L-Y-L-V-(C)-G-E	1143,5486	22	Cysteic acid (C)[19]
	A-L-(Y)-L-V-C-G-E-R-G-F*	1243,6345	16	Oxidation (Y)[16]
	A-L-(Y)-L-V-C-G-E-R-G-(F)*	1259,6288	16	Oxidation (Y)[16], Oxidation (F)[24]
	L-Y-L-V-C-G-E-R-G-F*	1156,6030	18	
	L-(Y)-L-V-C-G-E-R-G-F*	1172,5774	0	Oxidation (Y)[16]
	L-(Y)-L-V-(C)-G-E-R-G-F-F	1367,6512	16	Oxidation (Y)[16], Cysteic acid (C)[19]
	*G-E-R-G-(F)-F-Y-T-P-K-A	1288,6289	-2	Oxidation (F)[24]
	E-R-G-F-(F)-(Y)-T-P-K-A	1247,6011	-9	Oxidation (F)[25], Oxidation (Y)[26]
	E-R-G-(F)-F-Y-T-P-K-A	1231,6094	-1	Oxidation (F)[24]
	R-G-F-F-(Y)-T-P-K-A	1102,5597	-8	Oxidation (Y)[26]
	*G-F-F-Y-T-P-K-A	930,4919	21	
	*F-F-Y-T-P-K-A	873,4616	13	



Monoglycated insulin

(30days, non-reducing conditions)

		Observed mass	Match error PPM	Modification
4h	*G-E-R-G-F-F-Y-T-P-K-A	1272,6375	0	
	*G-E-R-G-F-F-(Y)-T-P-K-A	1288,6430	8	Oxidation (Y)[26]
	E-R-G-F-F-Y-T-P-K-A	1215,6085	-6	
	E-R-G-F-F-(Y)-T-P-K-A	1231,5897	-17	Oxidation (Y)[26]
	R-G-F-F-Y-T-P-K-A	1086,5537	-18	
24h	*G-E-R-G-(F)-(F)-Y-T-P-K-A	1288,6196	-10	Oxidation (F)[24 or 25]
	E-R-G-F-(F)-(Y)-T-P-K-A	1231,5885	-18	Oxidation (F)[24] or Oxidation (Y)[26]
	R-G-F-(F)-(Y)-T-P-K-A	1102,5513	-15	Oxidation (F)[24] or Oxidation (Y)[26]
	*G-F-F-Y-T-P-K-A	930,4513	-22	
48h	*S-(H)-L-V-E-A-L-(Y)-L-V-(C)*	1342,6448	0	Oxidation (H)[10], Dioxidation (Y)[16], Cysteic acid (C)[19]
	S-H-L-V-E-A-L	768,4381	16	
	A-L-(Y)-L-V-C-G-E-R-G-F*	1243,6317	14	Oxidation (Y)[16]
	A-L-(Y)-L-V-C-G-E-R-G-(F)*	1259,6217	10	Oxidation (Y)[16], Oxidation (F)[24]
	*V-C-G-E-R-G-F-F-Y-T-P-K-A	1636,7318	-22	Glycation (K)[29]
	*V-C-G-E-R-G-(F)-(F)-Y-T-P-K-A	1652,7360	-16	Glycation (K)[29], Oxidation (F)[24 or 25]
	*C-G-E-R-G-F-F-Y-T-P-K-A	1375,6392	-6	
	E-R-G-F-(F)-(Y)-T-P-K-A	1247,6010	-9	Oxidation (F)[25], Oxidation (Y)[26]
	E-R-G-(F)-F-Y-T-P-K-A	1231,6155	4	Oxidation (F)[24]
	R-G-(F)-F-Y-T-P-K-A	1102,5781	9	Oxidation (F)[25]
	R-G-F-F-Y-T-P-K-A	1248,6349	7	Glycation (K)[29]

Fig. 1 Oxidative modifications found in native and monoglycated insulin produced under non-reducing conditions (NRed1g). Peptide sequence aligned with the entire insulin chain, observed sequence mass and corresponding error (ppm), and assigned oxidative modification, with its respective location within the sequence, are shown. *Circles* indicate oxidized residues and *asterisks* highlight the corresponding sites of oxidative cleavage

mass modifications applied to all possible insulin residues (Table 1), parent tolerance of 200 ppm, and fragments tolerance of 0.3 Da. Manual verification of Protein Prospector results was performed by use of Data Explorer software v4 (Applied Biosystems). Matched sequences were validated if all major peaks in the MS–MS spectrum were explained by the candidate sequence and the spectrum contained peaks to confirm the peptide's modification.

Results and discussion

Insulin oxidation

In this work our objective was to investigate the oxidative behavior of glycated insulin with the determination of site-specific oxidative damage of both native and glycated insulin. The first step involved the in-vitro glycation of insulin and has been described previously [33]. All four of

A chain G-I-V-E-Q-C-C-A-S-V-C-S-L-Y-Q-L-E-N-Y-C-N

B chain F-V-N-Q-H-L-C-G-S-H-L-V-E-A-L-Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A

Monoglycated insulin

(reducing conditions)

		Observed mass	Match error PPM	Modification
4h	*G-E-R-G-F(FY)T-P-K-A	1288,6277	-3	Oxidation (F)[25] or Oxidation (Y)[26]
	E-R-G-F(FY)T-P-K-A	1231,6066	-3	Oxidation (F)[25] or Oxidation (Y)[26]
24h	*G-E-R-G-F-F-Y-T-P-K-A	1436,6795	-19	Glycation (K)[29]
	*G-E-R-G(FF)Y-T-P-K-A	1288,6524	16	Oxidation (F)[24 or 25]
	E-R-G-F-F-Y-T-P-K-A	1379,6578	-19	Glycation (K)[29]
	E-R-G-F(FY)T-P-K-A	1231,5986	-10	Oxidation (F)[25] or Oxidation (Y)[26]
	R-G-F-F-Y-T-P-K-A	1250,6138	-23	Glycation (K)[29]
	R-G-F(FY)T-P-K-A	1102,5420	-24	Oxidation (F)[25] or Oxidation (Y)[26]
	R-G-F-F-Y-T-P-K-A	1086,5533	-18	
48h	F-V-N-Q-H-L-C-G-S-H-L-V-E	1482,6821	-23	
	F-V-N-Q(H)(LC)(G)S-H-L-V-E	1558,6913	20	Oxidation (H)[5], Cysteic acid (C)[7] and Oxidation (G)[8]
	F-V-N-Q-H-L-C-G-S-H-L*	1254,6332	22	
	C-G-S-H-L-V-E-A-L-Y-L-V-C	1406,6578	-16	
	S-H-L-V-E-A-L-Y-L-V-(C)	1294,6186	-13	Cysteic acid (C)[19]
	*H-L-V-E-A-L-Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A	2440,2503	1	
	L-Y-L-V-C-G-E-R-G-F-F-Y	1466,7141	1	
	*(Y)L-V-C-G-E-R-G-F-F(Y)	1385,6453	19	Oxidation (Y) [16,26]
	*Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A	1914,9156	-8	Glycation (K)[29]
	*Y-L-V-C-G-E-R-G-F-F-Y-T(P)K-A	1764,8707	16	Pro->pyro-glutamic acid (P)[28]
	*Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A	1750,8680	3	
	L-V-C-G-E-R-G-F-F-Y-T-P-K-A	1587,7998	0	
	*V-C-G-E-R-G-F-F-Y-T(P)K-A	1652,7327	-18	Pro->pyro-glutamic acid (P)[28], Glycation (K)[29]
	*V-C-G-E-R-G-F-F-Y-T-P-K-A	1638,7721	-7	Glycation (K)[29]
	*V-C-G-E-R-G-F-F-Y-T-P-K-A	1474,7166	1	
	*C-G-E-R-G-F-F-Y-T-P-K-A	1375,6678	15	
	C-G-E-R-G-F-F(Y)T(P)K	1336,6272	21	Oxidation (Y)[26] and Oxidation (P)[28]
	C-G-E-R-G-F-F(Y)T(P)K	1320,6260	17	Oxidation (Y)[26] or Oxidation (P)[28]
	C-G-E-R-G-F-F-Y-T-P-K	1304,6233	10	
	(C)G-E-R-G-F-F(Y)T(P)	1240,5107	14	Cysteic acid (C)[19], Oxidation (Y)[26] or Oxidation (P)[28]
	G-E-R-G(F)F-Y-T-P-K-A	1452,7024	1	Glycation (K)[29] and Oxidation (F)[24]
	G-E-R-G-F-F-Y-T(P)K-A	1450,7169	22	Glycation (K)[29] and Pro->pyro-glutamic acid (P)[28]
	G-E-R(GFF)Y-T(P)K-A	1332,6031	13	Oxidation (G)[23], Oxidation (F)[24,25] and Pro->pyro-glutamic acid (P)[28]
	G-E-R(GF)F-Y-T(P)K-A	1316,6113	16	Oxidation (G)[23], Oxidation (F)[25] and Pro->pyro-glutamic acid (P)[28]
	(G)E-R-G-F-F-Y-T(P)K-A	1314,5503	-19	Oxidation (G)[20] and Pro->pyrrolidone (P)[28]
	G-E-R-G-F(F)Y-T(P)K-A	1302,6342	18	Oxidation (F)[25] and Pro->pyro-glutamic acid (P)[28]
	G-E-R-G-F-F(Y)T(P)K-A	1300,5855	-8	Oxidation (Y)[26] and Pro->pyro-glutamic acid (P)[28]
	G-E-R-G-F-F-Y-T(P)K-A	1286,6444	21	Pro->pyro-glutamic acid (P)[28]
	G-E-R-G-F-F-Y-T-P-K	1365,6761	5	Glycation (K)[29]
	G-E-R-G-F-F-Y-T-P-K	1201,6165	13	
	*G-E-R-G-F-F-Y-T-P *	1073,5292	22	
	E-R-G-F-F-Y-T(P)K-A	1231,6012	-8	Oxidation (P)[28]

Fig. 2 Oxidative modifications found in monoglycated insulin produced under reducing conditions (Red1g). Peptide sequence aligned with the entire insulin chain, observed sequence mass and corresponding error (ppm), and assigned oxidative modification, with its respective location within the sequence, are shown. *Circles* indicate oxidized residues and *asterisks* highlight the corresponding sites of oxidative cleavage

the glycated samples characterized in that study were subjected to oxidative stress in this work. The first sample was obtained in the absence of reducing agent (denoted "NRed1g" in the Experimental section) and was characterized as a monoglycated form of insulin with the glucose adduct located either at Lys-29 or Phe-1 (both residues from chain B), with these two monoglycated species coexisting. The other three samples were obtained under reducing conditions (excess $NaBH_3CN$), with the production of monoglycated, diglycated, and triglycated species (denoted "Red1g", "Red2g", and "Red3g", respectively, in the Experimental section). In this case, glycation occurs again at positions Phe-1 and Lys-29 of chain B, and also at position Gly-1 of chain A. Under reducing conditions there is also coexistence of monoglycated and diglycated glycated forms of insulin. The monoglycated species is glycated at Lys-29 or Phe-1, likewise the monoglycated insulin produced under non-reducing conditions. The diglycated species coexist in two isoforms, one glycated at Phe-1 and Lys-29 of chain B and the other glycated at Lys-29 of chain B and Gly-1 of chain A.

The next step of our work focused on studying the oxidative modifications of insulin, both native and glycated, using an MS-based approach. Oxidative damage was induced by use of a Fenton oxidation in-vitro system, as described in the Experimental section, and oxidized samples were analyzed after different periods of time (0, 4, 24, and 48 h). Preliminary experiments were followed up by analyzing an aliquot of the reactive mixture. These aliquots were analyzed by MALDI-MS, in both the reflectron and linear modes. In the linear mode, we looked for higher masses that could correspond to cross-linking products. Apart from the signal corresponding to the insulin dimer, no other peaks were detected in all the samples. Also, with regard to control reactions for native and glycated forms of insulin, no oxidation products or fragmentation were observed in these control samples for all the studied incubation periods. Subsequently, nano-liquid chromatography was used to separate oxidized and non-oxidized insulin samples, and the corresponding digested samples. This approach enabled identification of oxidatively modified residues in all of the samples studied, native and glycated insulin, and identification of peptide sequences resulting from oxidative fragmentation. All modifications found in oxidized native and glycated insulin samples are available as supplemental data (ESM Table 2).

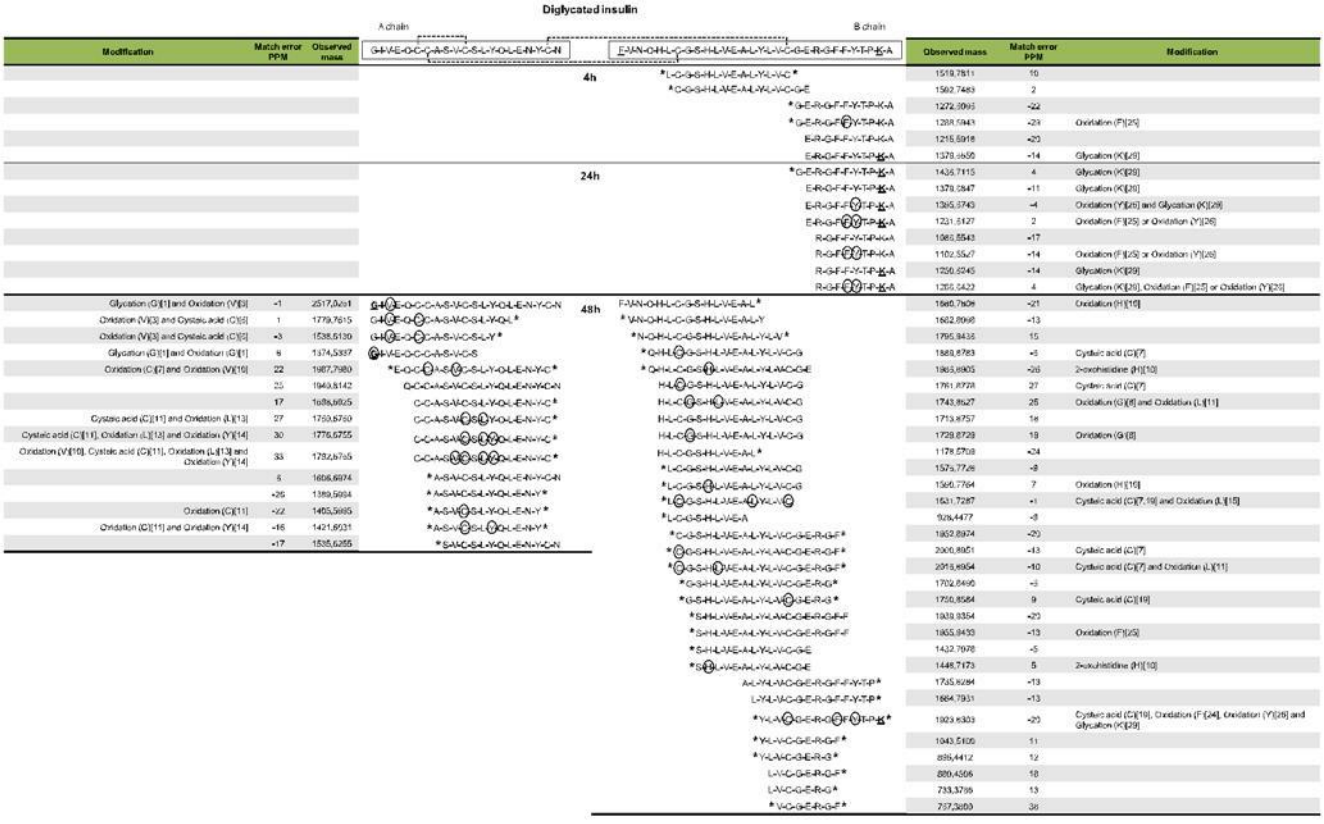
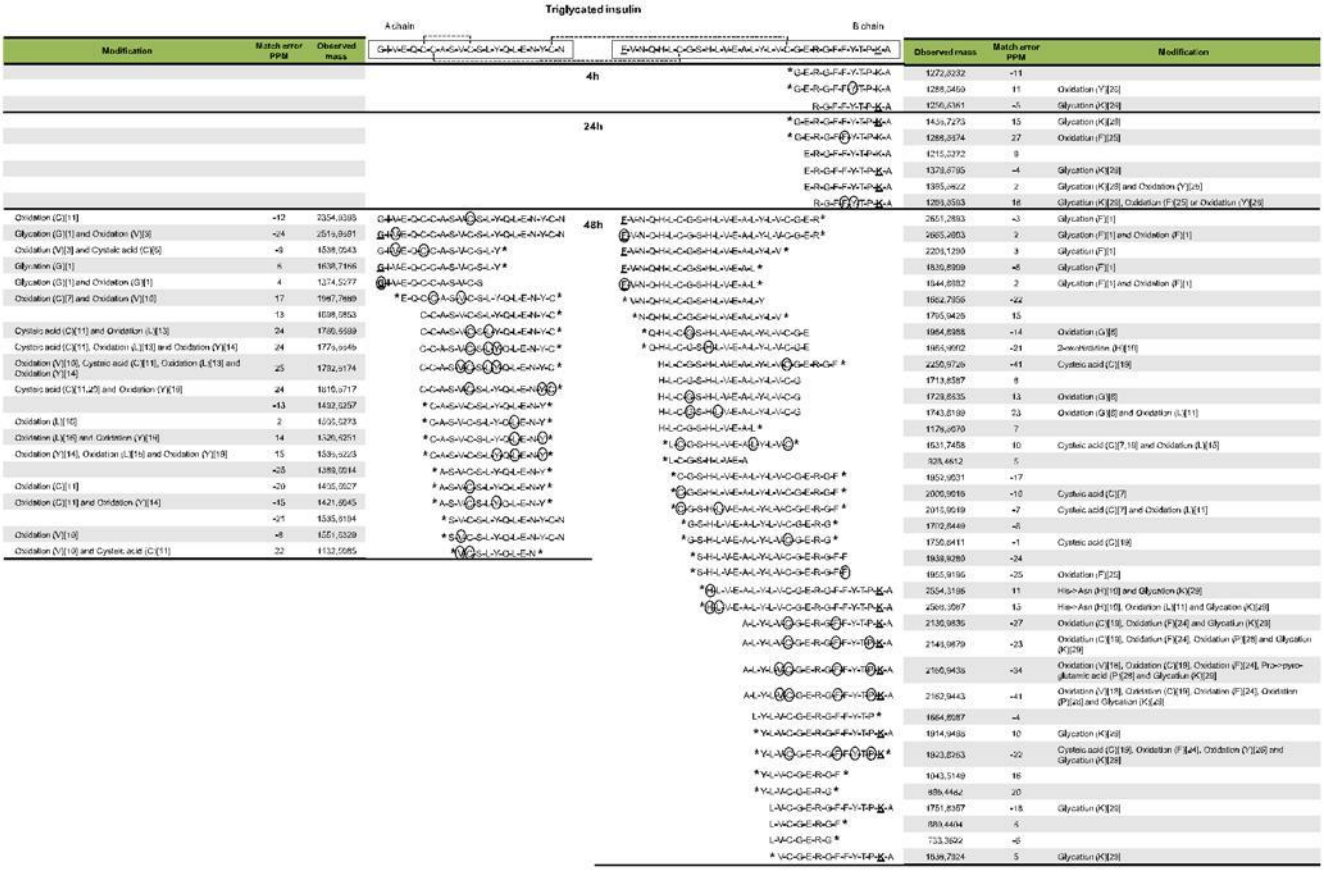


Fig. 3 Oxidative modifications found in diglycated insulin produced under reducing conditions (Red2g). Peptide sequence aligned with the entire insulin chain, observed sequence mass and corresponding error (ppm), and assigned oxidative modification, with its respective location within the sequence, are shown. *Circles* indicate oxidized residues and *asterisks* highlight the corresponding sites of oxidative cleavage

ESM Table 2 lists the peptide sequence, location of the modified residue within the sequence, and the masses observed for each peptide sequence with the corresponding associated error.

Oxidative modifications found in native and glycated samples (Figs. 1, 2, 3 and 4) result mainly from formation of hydroxyl and carbonyl derivatives, which is in conformity with the knowledge that the most frequent oxidation events result in formation of alcohol or aldehyde groups with associated mass increases of 16 or 14 Da, respectively [36]. The major affected residues are phenylalanine, tyrosine, proline, histidine, valine, leucine, and glycine. This outcome is in agreement with the literature, because these amino acids are known to be readily oxidized by MCO systems, besides being prone to any type of oxidative damage [4, 36]. In addition, we have also detected oxidation of cysteine residues to cysteic acid and oxidative conversion of histidine residue to aspargine. Although the rate of oxidative cleavage resulting mostly from hydroxyl radical abstraction of backbone Cα–H bonds is much slower than the initial abstraction of most side chains [37], we were still able to detect various products of backbone fragmentation. These products are marked as fragments from oxidative cleavage in Figs. 1, 2, 3 and 4 and are regarded as a result of oxidative cleavage because these peptide sequences are unique to oxidized samples and do not correspond to typical Glu-C cleavage sites. This oxidative cleavage occurred preferentially between the residues cysteine, glycine, phenylalanine, valine, leucine, and tyrosine in chain B, and between cysteine, valine, alanine, and tyrosine residues in chain A. Previous work also detected insulin oxidative fragmentation at these residues [34].

One of the objectives in this study was to perform a time-course evaluation of the oxidative modifications of insulin, in both its native and glycated forms, assessing the effect of glycation site on peptide oxidative behavior. In oxidized native insulin (Fig. 1) we detected one oxidatively modified residue, Tyr-26, and the formation of a B-chain C-terminus fragment containing this oxidized residue with the sequence between Gly-20 and Ala-30 after incubation for 4 h. We also observed ion peaks corresponding to the separated A and B chains. These results show that the initial oxidative damage takes place in the C-terminus of



Modification	Match error PPM	Observed mass
Oxidation (C)[11]	-12	2354,9085
Glycation (G)[1] and Oxidation (V)[3]	-24	2519,9681
Oxidation (V)[3] and Cysteic acid (C)[5]	-9	1538,0543
Glycation (G)[1]	8	1638,7186
Glycation (G)[1] and Oxidation (G)[1]	4	1274,5277
Oxidation (C)[7] and Oxidation (V)[10]	17	1997,7889
	13	1598,0853
Cysteic acid (C)[11] and Oxidation (L)[13]	24	1786,9599
Cysteic acid (C)[11], Oxidation (L)[13] and Oxidation (Y)[14]	24	1779,9946
Oxidation (V)[10], Cysteic acid (C)[11], Oxidation (L)[13] and Oxidation (Y)[14]	25	1782,9174
Cysteic acid (C)[11,20] and Oxidation (Y)[19]	24	1810,9717
	+13	1492,9257
Oxidation (L)[13]	2	1502,9293
Oxidation (L)[13] and Oxidation (Y)[14]	14	1526,9251
Oxidation (Y)[14], Oxidation (L)[13] and Oxidation (Y)[19]	15	1539,9223
	+28	1388,9514
Oxidation (C)[11]	-20	1435,9527
Oxidation (C)[11] and Oxidation (Y)[14]	-15	1421,9045
	+21	1535,9194
Oxidation (V)[10]	-8	1551,9329
Oxidation (V)[10] and Cysteic acid (C)[11]	22	1132,5685

Observed mass	Match error PPM	Modification
1272,6232	-11	
1288,5469	11	Oxidation (Y)[26]
1256,6161	-25	Glycation (K)[29]
1439,7275	15	Glycation (K)[29]
1288,5574	27	Oxidation (F)[25]
1215,5272	9	
1378,6795	-4	Glycation (K)[29]
1395,6822	2	Glycation (K)[29] and Oxidation (Y)[26]
1293,5503	16	Glycation (K)[29], Oxidation (F)[25] or Oxidation (Y)[26]
2651,2893	-3	Glycation (F)[1]
2665,2853	2	Glycation (F)[1] and Oxidation (F)[1]
2209,1290	3	Glycation (F)[1]
1835,8909	-6	Glycation (F)[1]
1844,8882	2	Glycation (F)[1] and Oxidation (F)[1]
1662,7856	-22	
1795,9426	15	
1964,8985	-14	Oxidation (G)[8]
1964,9962	-21	2-oxo-Histidine (H)[10]
2250,9726	-41	Cysteic acid (C)[19]
1713,8587	8	
1728,8535	13	Oxidation (G)[8]
1743,8189	23	Oxidation (G)[8] and Oxidation (L)[11]
1176,6070	7	
1531,7458	10	Cysteic acid (C)[7,19] and Oxidation (L)[15]
928,4612	5	
1952,9531	-17	
2009,9016	-10	Cysteic acid (C)[7]
2015,9569	-7	Cysteic acid (C)[7] and Oxidation (L)[11]
1712,8449	-8	
1750,8411	-1	Cysteic acid (C)[19]
1938,9280	-24	
1955,9196	-25	Oxidation (F)[25]
2554,3196	11	His->Asn (H)[10] and Glycation (K)[29]
2569,3587	13	His->Asn (H)[10], Oxidation (L)[11] and Glycation (K)[29]
2136,9836	-27	Oxidation (C)[19], Oxidation (F)[24] and Glycation (K)[29]
2143,9879	-23	Oxidation (C)[19], Oxidation (F)[24], Oxidation (P)[28] and Glycation (K)[29]
2160,9438	-34	Oxidation (V)[18], Oxidation (C)[19], Oxidation (F)[24], Pro->pyro-glutamic acid (P)[28] and Glycation (K)[29]
2182,9443	-41	Oxidation (V)[18], Oxidation (C)[19], Oxidation (F)[24], Oxidation (P)[28] and Glycation (K)[29]
1664,8087	-4	
1914,9488	10	Glycation (K)[29]
1963,8263	-22	Cysteic acid (C)[19], Oxidation (F)[24], Oxidation (Y)[26] and Glycation (K)[29]
1043,5149	16	
989,4462	20	
1751,8357	-18	Glycation (K)[29]
889,4494	6	
733,3692	-6	
1598,7824	5	Glycation (K)[29]

Fig. 4 Oxidative modifications found in triglycated insulin produced under reducing conditions (Red3g). Peptide sequence aligned with the entire insulin chain, observed sequence mass and corresponding error (ppm), and assigned oxidative modification, with its respective location within the sequence, are shown. *Circles* indicate oxidized residues and *asterisks* highlight the corresponding sites of oxidative cleavage

the B-chain, a tail-shaped and exposed region of the insulin molecule, and also near cysteine residues. Previous work examining peptide oxidation with hydroxyl radicals generated by X-ray radiolysis revealed that sulfur-containing residues, for example cysteines, are highly reactive, followed by aromatic residues (for example phenylalanine, tyrosine) [38, 39]. Our results are in total agreement because, after 24 h, neighboring residue Phe-25 appeared as oxidatively modified. A new fragment was also detected, belonging to a region near the N-terminus between Ser-9 and Leu-15, suggesting that oxidative damage spread to this more buried region because of previous disruption of the interchain disulfide bridge. Indeed, new fragments from the B-chain with sequences between Val-12 and Phe-25 were subsequently identified; these contained the oxidatively modified residues Tyr-16, Cys-19, and Phe-24. The results also show that "hotspot" cleavage sites occur between glycine residues and leucine (three fragments each), phenylalanine (two fragments), and cysteine, tyrosine, and valine (one fragment each).

Also in Fig. 1 we show an outline of the time-course of oxidative glycation of insulin under non-reducing conditions (NRed1g). This sample consists of a mixture of insulin glycated at Lys-29 or Phe-1. In general, the results show a very similar pattern along the time-course of oxidative damage when compared with oxidized native insulin. After 24 h, we detect the equivalent modified residues (Phe-25 and Tyr-26). Even so, at this time-point, Phe-24 was also seen as an oxidized residue and one additional fragment was identified (GFFYTPKA). After 48 h, we detect in this glycated form three other fragments with the peptide sequences SHLVEALYLVC, VCGERGFFYTPKA, and CGERGFFYTPKA; cleavage continues to occur preferentially between the residues cysteine, glycine, and valine. A newly oxidized residue of the B-chain, His-10, was also detected. The oxidation of His-10, not observed at the same time-point in oxidized native insulin, could be related to the glycated Lys-29, because these two residues are in close proximity in the 3D structure of insulin. Although we did not observe oxidation in the adjacent residues of Lys-29 (for

Time	Oxidized Sample	Oxidized residues Pro	Val	Leu	Gly	His	Cys	Tyr	Phe
4h	Native							25%	
	NRed1g							25%	
	Red1g							25%	33%
	Red2g								33%
	Red3g							25%	
24h	Native								33%
	NRed1g							25%	33%
	Red1g							25%	67%
	Red2g							25%	33%
	Red3g							25%	33%
48h	Native						33%	50%	67%
	NRed1g					50%	17%	50%	67%
	Red1g	100%			100%	50%	33%	50%	67%
	Red2g		40%	50%	67%	50%	83%	50%	33%
	Red3g	100%	40%	67%	67%	50%	100%	75%	100%

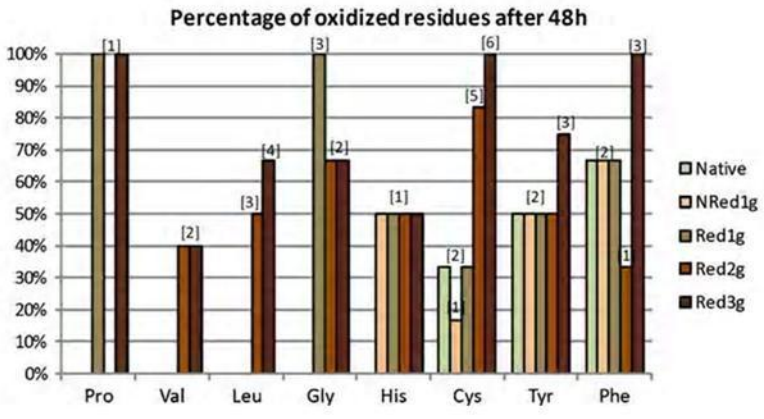
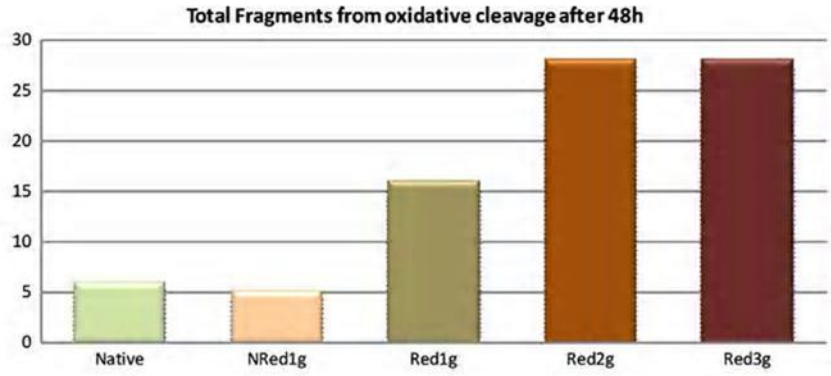


Fig. 5 Percentage of modified residues along the time-course of the oxidative damage for each sample. The percentage of oxidized residues for each sample after 48 h of oxidative damage is shown graphically. *Numbers in brackets* represent the number of residues that contribute to the correspondent percentage. *NRed1g*, monoglycated insulin produced under non-reducing conditions; *Red1g*, monoglycated insulin under reducing conditions; *Red2g*, diglycated insulin; *Red3g*, triglycated insulin

example Pro-28 and Ala-30), the His-10 oxidation is somewhat expected and in concurrence with previous work reporting the selective modification of histidine residues induced by active oxygen species in glycated insulin [34]. The authors correlated this observation with the site of glycation and consequent alteration in the steric conformation of the peptide.

Concerning the monoglycated insulin that resulted from the glycation process conducted under reducing conditions (Fig. 2, sample Red1g), we can observe a different and more complex result. These differences are seen after 48 h, when a greater number of fragments resulting from oxidative cleavage are detected, in a total of 13 different peptide sequences that do not result from Glu-C cleavage. The detected fragments all belong to the B-chain and comprise sequences throughout the entire chain, with "hotspot" cleavage sites occurring between the residues leucine and glycine (four fragments each) and cysteine and valine (three fragments each). We relate this observation with the two possible glycation sites in this chain (Phe-1 and Lys-29). As described in our previous paper [33], whereas monoglycated insulin under non-reducing conditions seems to be preferentially glycated at Lys-29, in monoglycated insulin under reducing conditions there is a similar abundance of the two possible glycation sites. This similar abundance is likely to be the reason why the oxidative damage covers all of the B-chain in this oxidized sample. Besides the additional cleavage sites in the residues lysine, proline, and histidine, six newly modified residues were identified: the other cysteine residue (Cys-7) and the other histidine residue (His-5) of the B-chain, both residues located in the vicinity of the glycated residue Phe-1; three glycines, with Gly-8 also close to the later residues, and Gly-20 and Gly-23 adjacent to the modified cysteine and phenylalanine residues; and Pro-28 adjacent to the glycated Lys-29.

When increasing the number of glycation sites in insulin, specifically in diglycated and triglycated insulin samples (Red2g and Red3g), the cumulative effect of glycation and oxidation processes in the insulin structure is very clear (Figs. 3 and 4, respectively). These major differences between the native and glycated forms of insulin, when submitted to in-vitro oxidative stress, are related to the formation of more radical species via autoxidation of the glycated forms of insulin, and the corresponding Amadori products that can result in the formation of the well-known advanced glycation end products (AGEs) [40–42]. In a variety of methodological approaches, changes caused by non-enzymatic glycation to protein structure, activity, and function have been studied [40, 43–50]. Herein, by use of the mass spectrometric approach described, we verify that glycation increases the oxidation-mediated structural damage revealed by determination of oxidative modification on oxidized glycated insulin. Both diglycated and triglycated samples are the only ones that show the highest oxidative damage through the higher number of cleavage sites and oxidatively modified residues in both the A and B-chains. In the case of B-chain, the results resemble those of the oxidized monoglycated insulin (under reducing conditions) with the oxidative damage occurring throughout the chain, although in Figs. 4 and 5 it is possible to see that more fragments are



Oxidized Sample at 48h	Frequency of cleavage sites after 48h of oxidative damage										
	Asn	Gln	Lys	Pro	His	Val	Phe	Tyr	Cys	Leu	Gly
Native						1	2		1	2	2
NRed1g						1	1	1	1	2	2
Red1g			1	1	1	2		1	2	4	2
Red2g	2	3	1	1		4	2	3	6	5	3
Red3g	3	3	1	1	2	5	2	4	6	5	4

Fig. 6 Summary of results from oxidative cleavage of native and glycated insulin. Total number of different fragments resulting from oxidative cleavage found in each sample after 48 h of oxidative damage is shown. The calculated frequency of cleavage sites after 48 h of oxidative damage of each sample is also given for each residue. This frequency reflects the number of different fragments resulting from oxidative cleavage at each residue. *NRed1g*, monoglycated insulin produced under non-reducing conditions; *Red1g*, monoglycated insulin under reducing conditions; *Red2g*, diglycated insulin; *Red3g*, triglycated insulin

formed not only in the vicinity of the already cited residues but also in the residues serine, asparagine, and glutamine. Moreover, other residues were identified as oxidatively modified: two leucines (Leu-11 and Leu-15) and the already glycated phenylalanine (Phe-1). In addition, we detected the formation of carbonyl derivatives of the residues glycine, leucine, and phenylalanine, and in proline (already observed in oxidized monoglycated insulin under reducing conditions). Oxidative damage observed in fragments resulting from the A-chain was only detected in diglycated and triglycated samples, which reflects the other glycation site at Gly-1 of A-chain. Results indicate the oxidative attack initiates in the region near the N-terminus, with modification of the residues cysteine (Cys-6 and Cys-11), valine (Val-3 and Val-10), tyrosine (Tyr-14), and leucine (Leu-13). These residues are also in close proximity to other modified residues from B-chain. Furthermore, the cleavage sites identified in the A-chain are prevalently located near the N-terminus region in the vicinity of cysteine and valine residues.

A summary of the results is shown in Figs. 5 and 6, which draw attention to oxidative damage comprising newly oxidized residues and cleavage sites, this damage being more severe as the number of glucose adducts in insulin increases. Figure 5 shows the percentage of modified residues along the time-course of oxidative damage for each sample; it is possible to observe the major differences occurring after 48 h. It is also worth noting that the oxidative damage involves newly oxidized proline, valine, leucine, histidine, and glycine residues only in glycated insulin samples. In Fig. 6 we summarize the results from oxidative cleavage of native and glycated insulin. The total number of different fragments resulting from oxidative cleavage found in each sample increases significantly after 48 h of oxidative damage. This increase is greater as the degree of insulin glycation increases. We also show the frequency of cleavage sites after 48 h of oxidative damage for each sample calculated for each residue. Cleavage sites resulting from oxidative damage occur preferentially between the residues phenylalanine, cysteine, glycine, leucine, valine, and tyrosine. In the more glycated forms of insulin, the frequency of these cleavage sites increases and new sites of oxidative cleavage appear in the residues histidine, proline, and lysine. In the more glycated samples, diglycated and triglycated insulin, oxidative damage also occurs in chain A, with residue oxidation and peptide cleavage taking place in the vicinity of cysteine bridges and glycation sites, affecting other residues, for example asparagine and glutamine.

In conclusion, the MS-based approach used in this work to study the time-course of oxidative modifications enabled investigation of the oxidative behavior of insulin in both its native and glycated forms. This MS-based approach enabled detection of more pronounced and earlier oxidative damage in glycated insulin, with the identification of newly oxidized residues and peptide fragmentation positions. Our results point out the correlation between the glycation sites and increased oxidation-mediated structural damage in the vicinity of these glycated residues.

Our data contribute to understanding the pro-oxidant behavior of glycated proteins, regarded as mediators in generating oxidative stress, by revealing the consequences of protein "glycoxidative" damage at a molecular level by use of the profitable combination of mass spectrometry and bioinformatics.

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ORIGINAL PAPER

Glycation and oxidation of histones H2B and H1: in vitro study and characterization by mass spectrometry

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Abstract Among the post-translational modifications, oxidation and glycation are of special interest, especially in diseases such as diabetes, and in aging. The synergistic interaction between glycation and oxidation, also known as "glycoxidation" is highly relevant due to its involvement in the production of deleterious changes at the molecular level. Non-enzymatic damage to nuclear proteins has potentially severe consequences for the maintenance of genomic integrity [54]. In this report, we study glycated histones and its in vitro oxidation. Data concerning the modifications that occurred in the histones were obtained by analysis of enzymatic digests (Glu-C and Arg-C) of unmodified and glycated histones, obtained before and after oxidation. Analysis was then performed using a MALDI-MS/MS-based approach combined with nano liquid chromatography. This approach allowed us to identify histone H2B and H1 specific-sites of oxidation and to distinguish the most affected residues for each histone. The results showed the occurrence of a cumulative effect of oxidative damage in the glycated histones when subjected to in vitro oxidation, suggesting that structural changes caused by glycation induces histones to a pro-oxidant state. Comparing the data of oxidized glycated histones with data from unmodified oxidized histones, using the same model of oxidation, the results clearly show that these oxidative modifications occur earlier and more extensively in glycated histones. Furthermore, the results pointed to an increased oxidative damage in the vicinity of the glycated residues.

Keywords Histones · Glycation · Protein oxidation · Mass spectrometry

Introduction

Intracellular and extracellular proteins are targets to a variety of non-enzymatic chemical modifications [1, 2], which can adversely affect function. The accumulation of these chemical modifications in long-lived proteins has been implicated in the pathophysiology of aging [3, 4] and a number of specific diseases, including diabetes [5–7] and Alzheimer's disease [8–10]. Among these chemical modifications, two inter-related protein modifications have received special attention: glycation and oxidation. Glycation is a complex multistep process that begins with the binding of reducing sugars to free amino groups of proteins, leading to Schiff base adducts [11–13] and aldoamines [14] as early glycation products. Through Amadori rearrangement reactions [15], ketoamine adducts are formed and because they are unstable compounds, a series of complex dehydration and oxidation reactions eventually leads to the formation of glycoxidation products termed AGEs [13].

These carbonyl products are known to be responsible for the propagation of oxidative damage initiated by protein glycation and mediating various pathophysiological processes, for example, in diabetes and aging. The autoxidation of protein-bound sugars represents one of the components of protein glycation that show the interrelation between glycation and oxidation [16, 17]. The other component of protein glycation is the "autoxidative

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glycosylation", in which radicals generated from free sugars interact with proteins. Both components are currently termed "glycoxidation" [17]. Recent work has demonstrated that radical fluxes associated with "glycoxidation" cause protein–carbonyl formation, fragmentation, and cross-linking [18, 19], generation of reactive long-lived protein-bound products [16], and the formation of oxidative adducts such as carboxymethyl-lysine and pentosidine [20]. However, data indicate that these oxidation products are formed preferentially from the oxidation of pre-glycated proteins. Moreover, previous studies have shown that pre-glycated and glycoxidized proteins can cause secondary damage on other biomolecules including enzymes, NADPH, and lipids [19, 21–25]. In the past years, various reports showed that histones are highly susceptible to glycation in the presence of several sugar compounds, such as glucose, fructose, ribose, and ADP-ribose [26]. These studies evidenced the formation of both pentosidine and total AGE fluorescence on glycated histones [27], the increase of AGE-histones in diabetic and aged rats [28], and the formation of ketoamine conjugates in the presence of ADP-ribose [29]. In a report by Wondrak and co-workers [25], the identification of a protein-bound free radical formed during early in vitro glycation of histone H1 was described.

It is known that histones, organized in nucleosomes, protect DNA from external damaging, such as oxidative damage. In fact, histones may represent the first target for glycation and consequent glycoxidation events in the nuclei, in order to protect the chemical integrity of DNA [25, 26, 30].

To date, most reports have focused attention on the study of histone post-translational modifications that influence the activity of histones, like acetylation, methylation, and phosphorylation [31–37]. In this study, we propose a mass spectrometry-based approach in the study of histones H1 and H2B "glycoxidative" damage. For this, we investigated the structural changes occurring in pre-glycated histones exposed to in vitro oxidative stress. We have identified specific-sites of glycoxidative modification, as well as, studied the time-course evaluation of these changes on both histones.

Experimental

Chemicals and reagents

Histone H2B (H4255) and TFA were acquired from Sigma (St. Louis, MO, USA). Histone H1 (382150) was acquired from Calbiochem (Merck, Darmstadt, Germany). MALDI matrices, α-cyano-4-hydroxy cinnamic acid (CHCA) and sinapinic acid, were supplied by Merck (Darmstadt, Germany). Toluene and acetonitrile (ACN) were from Riedel-deHaën (Buchs, Switzerland) and Labscan (Dublin, Ireland), respectively.

All chemicals were of analytical grade and milli-Q water was used throughout all experiments.

Histones in vitro glycation

In vitro glycation was carried out by incubating both histones with glucose in pseudo-physiological conditions. Histone H2B was acquired as a H2B-rich fraction and was purified on a C-18 analytical column (MRPC18, 2.1×5 cm, Agilent, US) [38]. Histone H2B (100 μg) and Histone H1 (500 μg) were incubated for 21 days at 37 °C with D-glucose 500 mM prepared in PBS 10 mM pH 7.4 with addition of toluene 5 mM as a bacteriostatic. Control reactions were performed simultaneously by incubating histone H2B and Histone H1 in the absence of glucose. All vials were degassed with N_2 prior to incubation. The glycation reaction was stopped by addition of acetic acid 0.5 M and the reaction mixture was dialyzed overnight against water, using a membrane (Spectra, VWR) with cut-off 3,500 Da. Control (H2B and H1 unmodified) and glycated (H2B_age and H1_age) samples were again concentrated on a Speed-vac, ressuspended in a final volume of 100 μL of milli-Q water and quantified with the Qubit assay (Invitrogen, US).

Histones in vitro oxidation

Unmodified and glycated histones (1 μM) were submitted to in vitro oxidation using two oxidation conditions: Ox1 and Ox2. In the case of the milder oxidation condition—Ox1—a typical oxidation reaction comprised, in PBS 10 mM pH 7.4, 1 μM of histone, 0.1 μM $FeCl_2$/EDTA and 0.2 μM H_2O_2. For Ox2 conditions, Fenton oxidant concentrations were 1 μM $FeCl_2$/EDTA and 2 μM H_2O_2. After various incubation periods (30 min, 1 h, 3 h and 24 h), the reactions were stopped with TFA 1% and, unless taken for further analysis, each sample was stored at −80 °C. In parallel, control reactions for both unmodified and glycated histones were performed by incubating histones only in phosphate buffer and $FeCl_2$/EDTA. All experiments were performed in duplicate.

Enzymatic digestion, nano-RP-HPLC and MALDI fractions collection

For in-solution digestion of control and oxidized histone H2B (unmodified and glycated), samples were initially diluted in NH_4HCO_3 25 mM pH 7.8. Endoproteinase Glu-C (Calbiochem, Merck, Darmstadt, Germany) solution was added in a final ratio of substrate:enzyme of 20:1 (*w*/*w*).

Digestion was performed in duplicate and samples incubated overnight at 25 °C. After digestion, samples were dried under vacuum in a Speed-vac and, unless taken for analysis, stored at −80 °C.

For in-solution digestion of control and oxidized histone H1 (unmodified and glycated), samples were diluted in Tris–HCl 100 mM/$CaCl_2$ 10 mM pH 7.6. Endoproteinase Arg-C (Roche, Switzerland) solution was added in a final ratio of substrate:enzyme of 50:1 (*w*/*w*). Digestion was performed according to manufacturer; duplicate and samples were incubated overnight at 37 °C. After digestion, samples were dried under vacuum in a Speed-vac and, unless taken for analysis, stored at −80 °C.

Digests of non-oxidized and oxidized histone samples (unmodified and glycated) were fractionated by liquid chromatography using an Ultimate 3000 (Lc Packings). For each sample, 1–2 μg of total protein were automatically injected onto a C18 trapping column (Zorbax 300SB-C18, 5 μm particle size, 5×0.3 mm, Agilent Technologies). Samples were washed over the trapping column for 3 min with a linear gradient of 95% eluent A (water, TFA 0.1%) and 5% eluent B (ACN 80%, TFA 0.04%) at a flow rate of 30 μL/min. After 3 min, the flow was redirected to the analytical column and the sample eluted onto a 150 mm×75 μm Zorbax 300SB capillary analytical C18 column (3.5 μm particle size, Agilent Technologies) at a flow rate of 0.3 μL/min. Separation of peptides occurred using a linear gradient of 5–15% eluent B over 10 min, 15–50% eluent B over 47 min, 50–90% eluent B over 2 min, followed by a 3-min hold at 90%. The peptides eluted from the monolithic capillary column were directly deposited onto 384-well MALDI plates, using a Probot (Lc Packings), together with simultaneously application of 170 nL of α-CHCA matrix solution. α-CHCA matrix solution was prepared by diluting 2.5 mg/mL of α-CHCA in ACN 70%/TFA 0.3% and adding 10 fmol of Glu-Fib as internal standard.

Mass spectrometry and database search

Peptide mass spectra were obtained with a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4,500 Da with ca. 1,500 laser shots. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems) which uses internal Mascot (Matrix Science) software for searching peptide mass fingerprints and MS/MS data. Bovine histone sequences were used for database search, up to two missed cleavage sites were allowed and for the search for oxidized peptides the Mascot software was updated through the UNIMOD web site (www.unimod.org) [39]. The database searches were performed using MS/MS data for each modification listed in Table 1, using the option of "variable modifications". With this option, it is possible to find matching peptides that are both modified and unmodified, as well as, it allows investigating more than one modifica-

Table 1 List of oxidative modifications updated from the UNIMOD web site through the mod_file of Mascot software

Modification	Description	Δ*m* (monoisotopic)
Amino (Tyr)	Tyrosine oxidation to 2-aminotyrosine	15.0108
Arg→GluSA	Arginine oxidation to glutamic semialdehyde	−43.0534
Cys→Oxoalanine	Cysteine oxidation to oxoalanine	−17.9928
His→Asn	Histidine oxidation to aspargine	−23.0159
His→Asp	Histidine oxidation to aspartic acid	−22.0319
Lys→Allysine	Lysine oxidation to aminoadipic semialdehyde	−1.0316
Lys→Aminoadipic acid	Lysine oxidation to α-aminoadipic acid	14.9632
Pro→Pyrrolidone	Proline oxidation to pyrrolidone	−27.9949
Pro→Pyrrolidinone	Proline oxidation to pyrrolidinone	−30.0105
Pro→Pyro-Glu	Proline oxidation to pyro-glutamic acid	13.9792
Trp→Hydroxykynurenin	Tryptofan oxidation to hydroxykynurenin	19.9898
Trp→Kynurenin	Tryptofan oxidation to Kynurenin	3.9949
Trp→Oxolactone	Tryptofan oxidation to oxolactone	13.9792
Trioxidation (Cys)	Cysteine oxidation to cysteic acid	47.9847
Oxidation, and dioxidation	Oxygen addition and hydroxylation (Lys, Arg, Cys,	15.9949
	Met, Tyr, His, Pro, Trp, Phe, Asp, Asn)	31.9898
Deamidation (Arg, Asn, Gln)		0.9840
Carbamylation (Lys, Arg, Cys, Met)	Isocyanate reaction with amino groups	43.0058
Quinone (Tyr)		29.9741

tion in the same residue within the same database search. No more than four variable modifications were combined in a single database search in order to prevent an increase in the number of false positive identifications. Positive identifications for oxidatively modified peptides were considered for individual ion scores above 50 with the default significance threshold, $p<0.05$. All modification sites were manually validated by an experienced investigator, using the Data Explorer software TM v4 (Applied Biosystems). Matched sequences and modified residues were validated if all major peaks in the MS/MS spectrum were explained by the candidate sequence and the spectrum contained peaks to confirm the peptide's modification.

Results and discussion

Histones glycation

After performing the in vitro histones glycation, histones molecular weight was monitored by MALDI-MS. After 21 days of incubation, a major band at *m/z* 14,877 in the case of histone H2B pointed out to 6 possible glycation sites (mass shift from underivatised protein of 1100), whereas in the case of histone H1, a major band at *m/z* 23,735 indicated 14 possible glycation sites (mass shift from underivatized protein of 2523; data not shown). To identify the glycation sites, unmodified and glycated samples were digested with endoproteinase Glu-C (histone H2B) and with endoproteinase Arg-C (histone H1), and the resultant peptides were fractionated by nano-flow reverse phase liquid chromatography and analyzed by tandem mass spectrometry (MALDI-MS/MS), as described in detail in the "Experimental" section. Two distinct enzymes were used for in-solution digestion of histones H2B and H1. The most suitable enzyme was chosen for each histone, based in its amino acid composition, in order to obtain resultant peptide fragments within a mass range appropriate to be analyzed by MALDI mass spectrometry and to obtain the best coverage rate. Positive identifications of glycated peptides that resulted from database search based on the neutral loss of 162 Da were subsequently verified by manual inspection of the MS/MS spectra. The characteristic fragmentation pattern of glycated peptides was distinguished, according to previous reports, and the peptide sequence with the assigned modification was confirmed through the *b*- and *y*-ion series in the mass spectrum [40–42]. Table 2 lists the glycation sites identified for histones H2B and H1, respectively. All of the identified glycated positions in both histones are lysine residues, which is in agreement with a previous report on glycation of histone H1 by Liebich and co-workers [43].

It should be noted that a particular lysine can be observed as glycated in one peptide sequence but it can be also observed as non glycated in the same sequence or in a partial overlapping sequence. Thus, as expected, the average number and position of glycates lysines can be different from molecule to molecule and that not all molecules present in a given sample contain all the identified glycated lysines.

Histones oxidation

The next step of our study focused on investigating histones modifications using a MS-based approach. As depicted in

Table 2 Glycation sites identified in glycated histones H2B and H1

Position	Sequences with observed glycation sites	Observed mass	Modified Lys
H2B			
5–23	AKSAPAP**K**KGS**K**KAVTKAQ	2220.2505	12; 16
5–23	AKSAPAP**KK**GSKKAVTKAQ	2220.2476	12; 13
35–41	**K**ESYSVY	1037.4603	35
100–114	RLLLPGELA**K**HAVSE	1794.9799	109
115–116	GT**K**AVTKYTSSK	1432.7509	117
H1			
5–25	APAAPAAAPPAEKTPV**KKK**AA	2487.3401	21; 22; 23
9–32	PAAAPPAEKTPVKKKAAK**K**PAGAR	2545.4105	27
128–140	PKKAAGAA**K**KTKK	1520.9149	136
149–162	KTAKKTPK**K**AKKPA	1719.0308	157
168–172	KKVA**K**	735.4622	172
161–178	PAAAAVTKKVAKSP**K**KAK	1972.1338	175
182–201	PKKAAKSAAKAVKPKAA**K**P**K**	2342.4287	199; 201
191–203	KAVKPKAAKP**K**VA	1513.9228	201
196–201	KAA**K**PK	804.4822	199

Identified sequences, corresponding masses, positions in the protein sequence and the modified lysines are listed

the "Experimental" section, a MCO in vitro system was used and the oxidative modifications of unmodified and glycated histones were examined. Two oxidation conditions were used (Ox1 (0.2 μM H_2O_2) and Ox2 (2 μM H_2O_2)) and oxidized samples were analyzed at different periods of time (0, 0.5, 1, 3, and 24 h). Nano liquid chromatography was used to fractionate the enzymatic digests from control and oxidized samples, followed by MALDI-TOF/TOF analysis for peptide sequencing and identification by tandem mass spectrometry.

For histone H2B, protein sequence coverage increases with glycation, rising from 63% in its unmodified form to 88% in its glycated form (Table 3). In the case of histone H1, protein sequence coverage increases from 33% in the unmodified form to 62% when glycated (Table 3). Similarly, protein sequence coverage also increases with oxidation (Table 3). The increase is more significant when stronger oxidant conditions are used (Ox2), as reported previously [44]. This is due, most probably, to the oxidative stress-induced alterations in protein conformation, allowing a better access of the enzyme to the cleavage sites of the protein, although oxidation could, in theory, block the enzyme recognition of cleavage sites. In that case, oxidation could reduce the coverage percentage. Assuming the increase of coverage rate reflects important changes in protein conformation, these results indicate that both glycation and oxidation promote significant conformational changes in these proteins, thus predictably affecting their function.

Oxidative-related modifications were found in the samples submitted to oxidative damage and are available as Electronic Supplementary Material Table S1.

Oxidative modifications found in unmodified and glycated samples (Figs. 1, 2, 3 and 4) result mainly from hydroxyl and carbonyl derivatives formation. Our results show oxidized side-chains of glycine, lysine, arginine, and proline residues yielding carbonyl derivatives, such as aminoadipic semialdehyde and α-amino adipic acid (Lys), glutamic semialdehyde (Arg and Pro), and pyro-glutamic acid, pyrrolidinone and pyrrolidone (Pro) [45–47]. In the case of oxidized histone H2B (Fig. 1), we also found histidine oxidation to aspartic acid and aspargine residues [45] and tyrosine oxidation to hydroxyphenylalanine [48]. In addition, we detected in oxidized lysine and arginine residues a post-translational modification of proteins (carbamylation) that results from the binding of isocyanate to amino groups, the occurrence of protein backbone cleavage in consequence of oxidative damage [44].

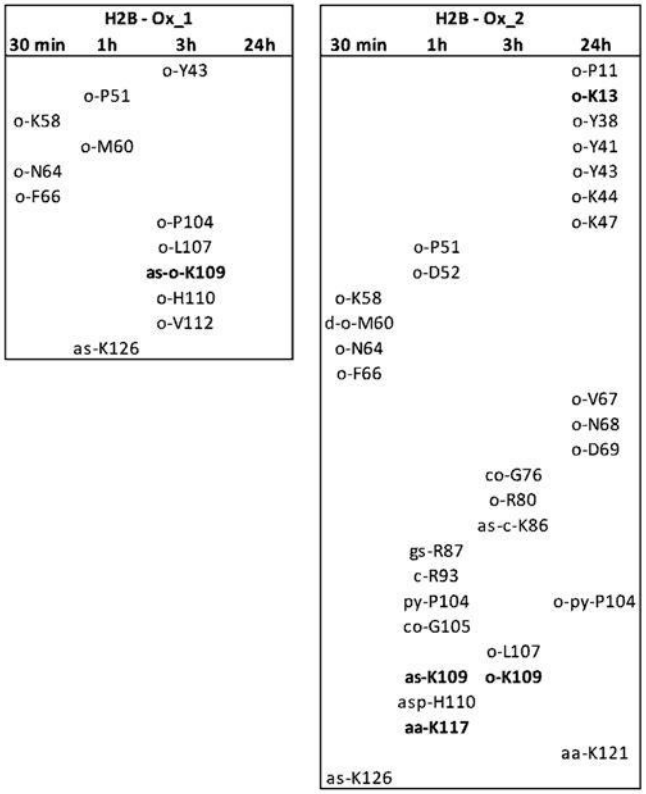
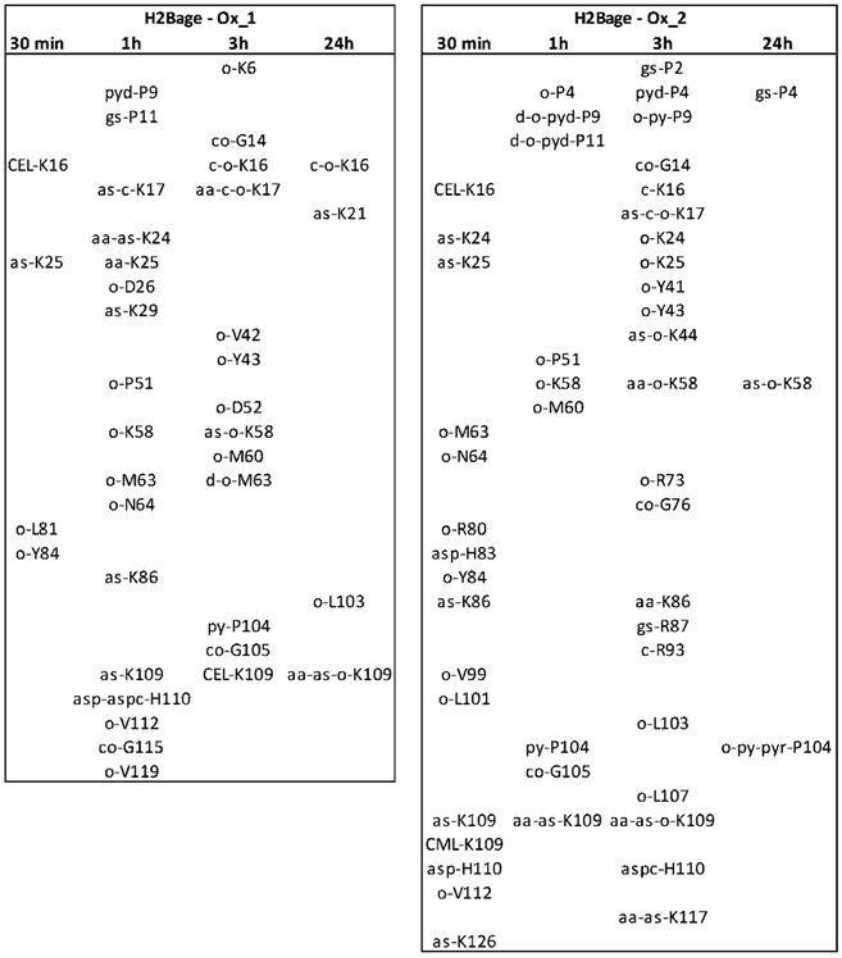


Fig. 1 Oxidative modifications found in unmodified H2B samples after 30 min, 1 h, 3 h, and 24 h of oxidation with Ox1 and Ox2 conditions. Only newly oxidized residues are shown along the time-course of oxidation. Residues are shown in one letter form with the number locating the residue within the H2B sequence. Before each residue, there is a letter code that classifies the oxidative modifications. *aa* lysine oxidation to α-aminoadipic acid; *as* lysine oxidation to α-aminoadipic semialdehyde; *asp* histidine oxidative conversion to aspargine; *aspc* histidine oxidative conversion to aspartic acid; *c* carbamylation; *co* carbonyl group formation; *d* dioxidation; *s* oxidation to glutamic semialdehyde in Arg or Pro residues; *o* oxidation; *py* proline oxidation to pyro-glutamic acid; *pyd* proline oxidation to pyrrolidinone; *pyyr* proline oxidation to pyrrolidone. *Residues in bold* correspond to glycated lysines identified in glycated H2B samples

Table 3 Percentage of sequence coverage for histones H2B and H1 in their unmodified and glycated forms, and when submitted to oxidative damage

Time		0x1	0x2		0x1	0x2
0	H2B	63		H2BAge	88	
0,5 h		81	67		84	92
1 h		79	87		94	96
3 h		70	87		98	98
24 h		70	89		98	98
0	H1	33		H1Age	62	
0,5 h		33	42		44	44
1 h		50	70		65	75
3 h		53	58		78	82
24 h		65	82		70	83

Data is shown for all oxidant conditions and along the time-course of the oxidative damage



H2Bage - Ox_1			
30 min	1h	3h	24h
		o-K6	
	pyd-P9		
	gs-P11		
		co-G14	
CEL-K16		c-o-K16	c-o-K16
	as-c-K17	aa-c-o-K17	
			as-K21
	aa-as-K24		
as-K25	aa-K25		
	o-D26		
	as-K29		
		o-V42	
		o-Y43	
	o-P51		
		o-D52	
	o-K58	as-o-K58	
		o-M60	
	o-M63	d-o-M63	
	o-N64		
o-L81			
o-Y84			
	as-K86		
			o-L103
		py-P104	
		co-G105	
	as-K109	CEL-K109	aa-as-o-K109
	asp-aspc-H110		
	o-V112		
	co-G115		
	o-V119		

H2Bage - Ox_2			
30 min	1h	3h	24h
		gs-P2	
	o-P4	pyd-P4	gs-P4
	d-o-pyd-P9	o-py-P9	
	d-o-pyd-P11		
		co-G14	
CEL-K16		c-K16	
		as-c-o-K17	
as-K24		o-K24	
as-K25		o-K25	
		o-Y41	
		o-Y43	
		as-o-K44	
	o-P51		
	o-K58	aa-o-K58	as-o-K58
	o-M60		
o-M63			
o-N64			
		o-R73	
		co-G76	
o-R80			
asp-H83			
o-Y84			
as-K86		aa-K86	
		gs-R87	
		c-R93	
o-V99			
o-L101			
		o-L103	
	py-P104		o-py-pyr-P104
	co-G105		
		o-L107	
as-K109	aa-as-K109	aa-as-o-K109	
CML-K109			
asp-H110		aspc-H110	
o-V112			
		aa-as-K117	
as-K126			

Fig. 2 Oxidative modifications found in glycated H2B samples after 30 min, 1 h, 3 h, and 24 h of oxidation with Ox1 and Ox2 conditions. Only newly oxidized residues or modifications are shown along the time-course of oxidation. Residues are shown in one letter form with the number locating the residue within the H2B sequence. Before each residue there is a letter code that classifies the oxidative modifications. *aa* lysine oxidation to α-aminoadipic acid; *as* lysine oxidation to α-aminoadipic semialdehyde; *asp* histidine oxidative conversion to aspargine; *aspc* histidine oxidative conversion to aspartic acid; *c* carbamylation; *co* carbonyl group formation; *d* dioxidation; *s* oxidation to glutamic semialdehyde in Arg or Pro residues; *o* oxidation; *py* proline oxidation to pyro-glutamic acid; *pyd* proline oxidation to pyrrolidinone; *pyyr* proline oxidation to pyrrolidone; *CML* carboxymethyl-lysine; *CEL* carboxyethyl-lysine

Histone H2B

In the control samples of histone H2B, we detect a single modified residue, an oxidized Met-63 (supplementary Table 1). When submitted to oxidative damage, the major affected residues are methionine, phenylalanine, proline, lysine, histidine, glycine, asparagine, and aspartic acid (Fig. 1). With the exception of residues such as asparagine and aspartic acid, this is in agreement with the literature [47, 49, 50].

With the milder oxidant conditions (Ox1), after 30 min, along with the oxidized Met-63, we detect the oxidation of Lys-58, Asn-64, and Phe-66 (Fig. 1). All these residues are located in the vicinity of Met-63, suggesting this is the region where oxidative damages initiates. At the same time-point, when using Ox2 conditions, we also find all methionine residues are oxidized and one more lysine residue—Lys-126—located in the C-term region of H2B, is oxidized to aminoadipic semialdehyde (Fig. 1). After 1 h of oxidation, oxidative damage appears to spread to the C-term region of H2B, in a total of nine newly oxidized residues. This is also observed in the case of Ox2 conditions but, at this time-point (1 h), results from Ox1 conditions resemble the results of Ox2 conditions after only 30 min of oxidation. Also of notice with stronger oxidant conditions (Ox2), are the oxidative modifications of some residues that go beyond the initial hydroxyl derivative formation (glutamic semialdehyde in Arg-87, carbamylation of Arg-93, pyro-glutamic acid in Pro-104, asparagine conversion of His-110, formation of aminoadipic derivatives in Lys-109 and Lys-117). In Ox1 conditions, only longer incubation periods, 3 and 24 h, allows oxidative damage reach the C-term region of H2B (Pro-104, Leu-107, Lys-109, His-110, and Val-112). In contrast, with stronger oxidant conditions (Ox2), we identify oxidatively modified residues also in the N-term region of H2B in a total of 16 newly oxidized residues after 3 and 24 h of oxidation. It should be noted that, most probably due to differences in relative abundance, in all oxidation conditions, occasionally, few residues are detected as oxidatively modified at a given time-point on the oxidative damage and not detected in the following time-point.

When glycated and submitted to in vitro oxidative damage, histone H2B is markedly more susceptible to oxidation, as shown by data presented in Fig. 2. It clearly illustrates the higher number of oxidatively modified

H1 - Ox_1

30 min	1h	3h	24h
		gs-o-P6	
	py-P9	gs-o-py-P9	
	py-P13	o-py-P13	
	py-P14	o-py-pyr-P14	
		c-K21	
	c-K22		
	as-c-K23		
co-G-37			
py-P38			
		pyd-P39	
		c-K130	
		c-K136	
		as-o-K137	aa-K137
		as-o-K139	
		as-o-K140	
		co-G143	
			as-K148

H1 - Ox_2

30 min	1h	3h	24h
py-P6	d-py-P6	gs-P6	
py-P9	d-py-P9	gs-py-P9	
py-P13	d-py-P13	gs-py-P13	
o-py-P14	d-py-P14	pyd-P14	
c-K17			
pyd-P19			
c-K21			**as-c-K21**
c-K22		**as-c-K22**	
c-K23		**as-K23**	
	gs-P28		
	c-R32		
	o-R33		
	o-K34		
co-G-37			
py-P38			
	o-P39		
	o-V40		
	c-K52		
o-V57			
as-K63			
as-K64			
			aa-K97
	co-G98		
			co-G133
			as-K136
			aa-K137
			as-K139
			as-K140
	py-P182		
c-K184		aa-K184	
c-K187		aa-K187	
		as-K191	c-K191
	o-K194	aa-K194	
	py-pyr-P195		
	c-K196	aa-o-K196	
	c-K199	**aa-c-K199**	
	d-gs-o-py-P200		py-pyr-P200
o-K201	**aa-as-c-o-K201**		
		o-V202	
	as-K204	as-c-K204	
	py-P205		
	aa-K206		as-K206
			gs-P210
			aa-K211

Fig. 3 Oxidative modifications found in unmodified H1 samples after 30 min, 1 h, 3 h, and 24 h of oxidation with Ox1 and Ox2 conditions. Only newly oxidized residues are shown along the time-course of oxidation. Residues are shown in one letter form with the number locating the residue within the H1 sequence. Before each residue there is a letter code that classifies the oxidative modifications. *aa* lysine oxidation to α-aminoadipic acid; *as* lysine oxidation to α-aminoadipic semialdehyde; *asp* histidine oxidative conversion to aspargine; *aspc* histidine oxidative conversion to aspartic acid; *c* carbamylation; *co* carbonyl group formation; *d* dioxidation; *s* oxidation to glutamic semialdehyde in Arg or Pro residues; *o* oxidation; *py* proline oxidation to pyro-glutamic acid; *pyd* proline oxidation to pyrrolidinone; *pyyr* proline oxidation to pyrrolidone. Residues in *bold* correspond to glycated lysines identified in glycated H1 samples

residues and its occurrence in an earlier stage when compared to oxidized unmodified histone H2B. This comes in agreement with our previous study on the oxidative modifications of glycated insulin [51]. So, this outcome is most probably due to changes in protein conformation by glycation, but also by increasing oxidation susceptibility.

Regarding the glycated H2B control samples, we detect three oxidatively modified residues (Phe-66, Phe-71, and Asn-68 (supplementary Table 1)). Although not located right in the vicinity of glycated lysines, these residues are in the secondary structure near to the two terminal regions of H2B containing glycated lysines (the vicinity of the glycation sites was considered spatially rather than sequen-

H1age - Ox_1			
30 min	1h	3h	24h
		gs-P6	
		gs-P9	
py-P13	pyr-P13	o-py-P13	
o-py-P14		o-py-pyd-P14	
as-K17	as-o-K17		
d-P19		o-py-pyd-P19	
CML-K21	aa-c-K21		
CML-K22	aa-c-K22		
	aa-as-K23		
aa-o-K26	as-c-o-K26		
	aa-K27	o-K27	
	py-P28	pyd-P28	
		o-R32	
		gs-R33	
o-K34			
co-G37			
py-P38	gs-py-P38	o-py-P38	d-o-py-P38
py-P39	pyd-P39		o-py-P39
		o-V40	
		o-K46	
	c-K52		
	aa-c-K63		
	as-c-K64		aa-c-K64
	CML-K75		
	d-F105		
		aa-K140	
		co-G143	
		gs-P147	
		as-K148	
			as-K149
		as-K152	
		as-K153	
		o-V170	
		aa-K172	
		aa-K176	
		aa-K178	
c-K181	as-K181	aa-K181	
as-K183			
as-K184			
	c-K187		
			gs-P195
		o-K196	aa-K196
CML-K199		o-K199	
pyr-P200		o-P200	gs-o-py-P200
		o-K201	aa-K201
		o-V202	
			o-K204
py-P205		pyd-P205	py-pyr-P205

H1age - Ox_2			
30 min	1h	3h	24h
		d-P6	
d-P9			
o-py-pyr-P13	d-o-py-pyd-P13		
gs-py-P14	gs-o-pyr-P14		
aa-as-K17	c-o-K17		
d-pyd-P19	pyr-P19	o-py-P19	
CML-aa-K21	aa-o-K21	aa-c-o-K21	
CML-aa-K22	aa-c-K22	aa-c-o-K22	as-c-K22
	aa-as-K23	o-K23	as-c-K23
aa-as-o-K26			
o-K27	CML-K27	aa-o-K27	
d-py-P28	o-P28		
co-G30			
		gs-R32	
gs-R33			
aa-as-c-o-K34			
co-G37			
d-P38	py-P38		
py-P39			
		c-K52	
	aa-K63		
	c-K64		
CML-K75			
d-F105			
		aa-K129	
		as-o-K130	
		as-o-K137	
		o-K139	
		aa-K148	
	aa-K149		
		aa-K152	o-K152
			o-K153
		gs-P155	
	as-K156		as-o-K156
		as-K157	
	aa-c-K159	aa-as-K159	
	aa-o-K160		aa-c-K160
	o-V166		
	o-K169		
	o-V170		
	as-K176		
	o-K178		
	o-K181		
	as-K184		
	py-P195		pyr-P195
aa-K196			
as-K199	CEL-K199		
	py-P200		
o-K201			as-o-K201
o-V202			
as-K204	aa-K204		
	py-P205		
	as-K206		
aa-K207	as-K207		

Fig. 4 Oxidative modifications found in glycated H1 samples after 30 min, 1 h, 3 h, and 24 h of oxidation with Ox1 and Ox2 conditions. Only newly oxidized residues are shown along the time-course of oxidation. Residues are shown in one letter form with the number locating the residue within the H1 sequence. Before each residue there is a letter code that classifies the oxidative modifications. *aa* lysine oxidation to α-aminoadipic acid; *as* lysine oxidation to α-aminoadipic semialdehyde; *asp* histidine oxidative conversion to asparagine; *aspc* histidine oxidative conversion to aspartic acid; *c* carbamylation; *co* carbonyl group formation; *d* dioxidation; *s* oxidation to glutamic semialdehyde in Arg or Pro residues; *o* oxidation; *py* proline oxidation to pyro-glutamic acid; *pyd* proline oxidation to pyrrolidinone; *pyyr* proline oxidation to pyrrolidone; *CML* carboxymethyllysine; *CEL* carboxyethyl-lysine

tially based on the information available online: http://www.uniprot.org/uniprot/P62808). Because these residues only appear as oxidatively modified in unmodified oxidized H2B samples, we suggest glycation as the responsible factor for this pro-oxidative behavior. Reactive species can be generated from autoxidation of either glucose or Amadori intermediates and diffuse to neighboring susceptible amino acid chains [52]. After oxidizing glycated H2B samples, we were able to identify the presence of carboxyethyl-lysine and carbomethyl-lysine in two lysines that were identified as glycated in the control samples. This modification was found for both oxidant conditions along

the time-course of oxidation and identified in both end terminals of H2B. In concurrence, our results show that for all oxidant conditions, oxidative damage occurs preferentially in the vicinity of the previously glycated lysines that are located in both end terminals of the protein. This can be observed in Ox1 oxidant conditions just after 1 h of oxidation. In these samples, in addition to identifying common oxidized residues with unmodified H2B, we detect the modification of various residues in the N-term region and residues in C-term region, all located in the proximity of glycated lysines. With Ox1 conditions and after 3 and 24 h of incubation, oxidative damage reaches a few more residues located near the pre-existent ones. A similar behavior is observed when stronger oxidant conditions are used (Ox2) although, as predictable, a higher number of modified residues are identified until the 3 h of oxidation. Newly modified residues are located in the region between amino acids 40–50 (lysine, tyrosine, and histidine residues), and in the region between amino acids 80–100 (arginine, tyrosine, and histidine residues). In the case of pre-glycated histone H2B, besides the higher number of oxidatively modified residues, there is also a higher occurrence of further oxidative modifications in residues such as lysine, proline, arginine, and glycine that have evolved from the initial hydroxyl derivative formation. This scenario occurs as early as after 1 h of oxidation with Ox1 oxidative conditions, contributing to the idea of the pro-oxidative behavior of glycated proteins.

Histone H1

With respect to unmodified histone H1, the major affected residues with oxidation are proline, glycine, lysine and valine residues (Fig. 3). Histone H1, currently called the linker histone, is a lysine-rich histone (28% of lysines) and thus, oxidation of lysine residues is expected. Although we also identified hydroxyl and carbonyl derivatives (as for oxidized H2B samples), in the case of oxidized H1 samples we observe much more products that are resultant from further oxidation of the initial oxidative event (hydroxylation). We identified the oxidative conversion of lysine residues to aminoadipic derivatives (acid and aldehyde), proline residues to pyro-glutamic acid and glutamic semialdehyde, pyrrolidinone, and pyrrolidone, and the oxidation of arginine residues to glutamic semialdehyde (Fig. 3).

In control samples, we detect a single modified residue, Gly-37 (supplementary Table 1). After 30 min of oxidation, with Ox1 oxidant conditions, we identify the carbonylated Gly-37, and the adjacent Pro-38 converted to pyro-glutamic acid. At this time-point, Ox2 oxidant conditions also induce oxidation of these residues, as well as proline and lysine residues located in the N-term region of H1 (Fig. 3). Prolines are mainly converted to pyro-glutamic acid while lysines are carbamylated and converted to aminoadipic semialdehyde derivatives. We further identified a modified valine in the N-term region and 3 modified lysines located in the C-term region of H1. These results point out to the N-term region of H1 as structurally more susceptible region for oxidative damage.

After 1 h of oxidation, for Ox1 conditions, results show that oxidative damage spreads within the N-term region of H1, similar to the results found after 30 min with Ox2 conditions (Fig. 3). In the case of Ox2 samples, principal modifications occur in residues located in the proximity of oxidized residues already identified.

For longer oxidation periods, 3 and 24 h, no damage to the C-term region of histone H1 is observed when using Ox1 conditions, while with Ox2, oxidative damage occurs in both terminal regions.

As observed for pre-glycated and oxidized histone H2B, histone H1 is more susceptible to oxidation when pre-glycated (Fig. 4). In fact, data shows (Figs. 3 and 4) a higher number of oxidatively modified residues and its occurrence in an earlier stage for glycated histone H1.

In glycated H1 control samples, we detect ten oxidatively modified residues that include proline and lysine residues. These residues are either next to or located in the vicinity of glycated lysines (the vicinity of the glycation sites was considered spatially rather than sequentially based on the information available online: http://www.uniprot.org/uniprot/A3KN02). The pro-oxidative behavior of glycated histone H1 is supported by the identification of two lysines located in the N-term region (Lys-21 and Lys-22) modified to carboxymethyl-lysine. The susceptibility of neighboring residues comes as a consequence of reactive species generated from autoxidation events that occur after glucose adducts formation. Following oxidation, this pro-oxidative behavior is enhanced, in consistency with the previous results and cited literature [18–20, 53]. With oxidation, other glycation sites are identified as modified to carboxyethyl-lysine and carboxymethyl-lysine. These AGE-lysines are located in both end terminals of H1 and in the middle region of the protein, between 70 and 80. Thus, also for glycated histone H1, results show that for all oxidant conditions, oxidative damage prevails to occur in the vicinity of these previously glycated lysines. This can be observed for Ox1 oxidant conditions after 30 min of oxidation, by the identification of oxidized residues near glycated lysines (in N- and C-term regions and in the region between amino acids 130–140 (Fig. 4)). A higher number of oxidatively modified residues is observed after 3 h of oxidative damage, with 40 oxidized amino acids, of which 20 were not observed previously. These residues are mainly proline, lysine, valine, glycine and arginine. At this time-point, oxidative damage has spread through the entire

sequence, an outcome that is quite distinct from the correspondent unmodified H1 results.

Similar results are obtained when stronger oxidant conditions are used (Ox2) although, as predictable, a higher number of modified residues are identified and these identifications are seen at an earlier stage in the time-course of oxidation (Fig. 4). For this condition (Ox2), a similar damage to glycated histone H1 is observed just after 30 min and 1 h of incubation. Newly oxidized residues include lysine and valine located mainly in the region 140–180.

Conclusions

Our results point out to well-defined correlation between protein glycation and an increased oxidation-mediated structural damage in the vicinity of glycated residues. The MS-based approach used in this report allowed to perform a time-course study of oxidative modifications of histones H2B and H1, as well as, to further investigate the oxidative behavior of both histones when glycated. Results of unmodified histone samples incubated in the milder oxidant conditions (Ox1) showed that both histones were affected to a small extent, accounting for 7% of total oxidative damage in the studied period of incubation. Furthermore, for unmodified samples in general, no newly modified residues were found after 3 h of oxidation. The percentage of total oxidative damage increased to a maximum of 19% and 13% for histone H2B and histone H1, respectively, when using the stronger oxidant conditions (Ox2).

The investigation of the oxidative behavior of glycated histones pointed out to a more pronounced and earlier occurrence of the oxidative damage with the identification of a higher number of oxidative modifications and oxidized residues. Both glycated histones showed a similar behavior, which was clearly observed with just the milder Ox1 oxidant conditions immediately after 30 min or 1 h of oxidation. In these conditions, total percentage of oxidized protein throughout the time-course of oxidation increased to a maximum of 16% and 19% for histone H2B and histone H1, respectively. When stronger oxidant conditions (Ox2) were employed, results showed an even higher contrast with the correspondent oxidized unmodified samples. Oxidative damage to glycated histones is much more severe, reaching total percentages of oxidized protein as high as 31% and 24% for histone H2B and histone H1, respectively. While in the case of unmodified samples, newly modified residues are found throughout the oxidation period of 24 h, for glycated samples the latter only occurs until the 3 h of oxidation. Moreover, in glycated samples, the total percentage of observed modified residues tends to decrease after 24 h of oxidation, which emphasizes the major oxidative damage to these samples with the occurrence of events such as the formation of cross-linking products that in turn lead to protein precipitation.

The efficient combination of mass spectrometry and bioinformatic software tools presented in this report allowed to study in important histone proteins the "glycoxidative" damage, in an effort to verify and better understand the pro-oxidant behavior of glycated proteins at the molecular level. Moreover, we consider this approach as an efficient way for carrying out the in vivo study of these modifications on histone proteins from biological samples in physiological conditions of interest.

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5. Discussion

5.1 Characterization of protein oxidative damage by mass spectrometry

There is no question that posttranslational modification of proteins by reactive oxygen and nitrogen species has been largely associated with a number of pathologies and biological aging (Stadtman and Berlett, 1997; Beal, 2002), due to alterations in the structural and functional integrity of specific proteins. However, controversy remains due to the problem of whether these posttranslational modifications truly cause the progression of disease or are only a part of the pathologic processes. An unambiguous answer to these questions requires a series of experiments beginning with the identification and quantification of the various posttranslational modifications that specific proteins accumulate *in vivo*, followed by their functional characterization. Afterwards it would be necessary to verify if the quantity of a given posttranslational modification is causally related to a specific pathology. Mass spectrometry appears as the technique of choice for the qualitative and quantitative detection of posttranslational protein modifications, since it allows as unambiguous assignment of the nature and location of the given modification. Nevertheless, challenges such as low abundance and hydrophobic proteins, protein turnover and repair, chemical instability of protein oxidation products, do not enable the study of *in vivo* oxidative posttranslational protein modifications to emerge at the same rate as the study of *in vitro* reactions of reactive oxygen and nitrogen species with proteins. Regardless the importance of validating *in vitro* data through experiments performed *in vivo*, important contributions with mechanistic studies and identification of 'novel' protein oxidation products have been made through *in vitro* model experiments. In order to further enlighten the issue regarding the alterations occurred during protein oxidative damage we initiated our study by performing a mass spectrometric analysis of BSA subjected to *in vitro* oxidative stress (Paper I). To monitor the degree of oxidative damage we firstly look at changes in protein carbonylation and by SDS-PAGE. Both approaches indicated us that oxidative damage of BSA was taking place in a time- and oxidant concentration-dependent. A significant increase in bound carbonyls was

observed in the case of oxidized samples which accompanied a correspondent decrease in the band intensities of the SDS gels when compared to native BSA samples. Both outcomes are in agreement with the literature (Traverso *et al.*, 1997; Ogasawara *et al.*, 2006) and with each other. Throughout the oxidation period, the fading of the native BSA band is compatible with the increase in the formation of carbonyl groups that are known to lead to protein cross-linking and precipitation (Berlett and Stadtman, 1997).

The next step focused on discovering structural changes in oxidized BSA by analyzing tryptic digests of oxidized and nonoxidized BSA by nano-liquid chromatography coupled with MALDI-TOF/TOF mass spectrometry. This methodology enabled us to sequence and to identify oxidized peptides using software Mascot for database search by including the modifications defined on the Unimod website (Creasy and Cottrell, 2004). We found residues such as cysteine, methionine, tryptophan, lysine, arginine, histidine, proline and tyrosine as the main affected residues by the oxidative damage. These findings showed to be in agreement with the literature that cites these amino acids as especially prone to oxidative damage and readily oxidized by metal-catalyzed oxidation systems (Stadtman and Levine, 2003; Guan and Chance, 2005). Also in agreement (Berlett and Stadtman, 1997; Shacter, 2000; Requena *et al.*, 2001; Stadtman and Levine, 2003) were the modifications found in specific residues such as the oxidation of the side-chains of lysine, arginine and proline to carbonyl derivatives like aminoadipic semialdehyde and α-amino adipic acid (Lys), glutamic semialdehyde (Arg) and pyrrolidinone (Pro); oxidation of cysteine to cysteic acid; oxidation of histidine to aspartic and asparagine; oxidation of tyrosine to amino-tyrosine and hydroxyphenylalanine; and formation of carbamyl group in lysine, cysteine and methionine. The later was considered to be a product of secondary reactions that indicated the occurrence of protein fragmentation. Besides determining the main affected residues and the type of oxidative modifications within the oxidized BSA structure, important information was obtained by performing a time-course evaluation until the two hours of oxidative damage. We observed that specific regions of BSA were preferentially affected but at different points in the time-course. In addition, the residues initially oxidized were mainly cysteine, methionine, lysine, arginine, proline and tyrosine residues located in surface exposed regions and near sections

containing cysteine residues. With the oxidation progress, we observed oxidative damage reaching residues that were most certainly previously shielded such as tyrosine, histidine, phenylalanine and aspartic acid. Because this type of residues were not identified as oxidatively modified in early stages and the number of newly identified oxidized residues did not show a linear behavior along the time-course, we suggested a point in time where albumin structure was no longer able to tolerate the oxidative alterations and the consequent conformational changes allowed a better accessibility for the oxidant species. Therefore, increased damage was observed throughout the protein sequence for longer periods and harsher oxidant conditions.

The next protein to be studied under similar conditions and by employing the same MS-based approach for the investigation of the oxidative modifications was insulin (Paper 3). Insulin was chosen as a 'smaller version' of BSA due to its smaller molecular weight, though still containing sensitive amino acids to oxidative damage and cysteine disulfide bridges. Unlike BSA, because of insulin's smaller molecular weight, we were able to separate oxidized and non-oxidized samples by nano-liquid chromatography before and after enzymatic digestion. This approach not only allowed us to identify oxidatively modified residues but also to identify peptide sequences resulting from oxidative fragmentation. As for the oxidized BSA results, oxidative modifications found in oxidized insulin samples resulted mainly from hydroxyl and carbonyl derivatives formation, with residues such as phenylalanine, tyrosine, proline, histidine, valine, leucine and glycine residues affected by the oxidative damage. In addition, we also detected the oxidation of cysteine residues to cysteic acid and the oxidative conversion of histidine residue to aspargine. When looking at the time-course evaluation of insulin oxidation, results also showed to be in conformity with what has been described so far. Oxidative damage to insulin initiated at the exposed region of the C-terminal of B-chain and also near cysteine bridges since, as early as this in the time-course of insulin oxidation, we were able to observe the separation of A and B chains. Further oxidation was observed closer to the N-terminal region which suggested that oxidative damage was able to spread to this more concealed region in spite of the interchain disulfide bridges disruption, somewhat what was observed for oxidized BSA.

With respect to protein fragmentation as a consequence of oxidation, in the case of oxidized insulin samples, we were able to detect various products of backbone fragmentation that represented peptide sequences unique to oxidized samples and without expected enzymatic cleavage sites. We observed that oxidative cleavage occurred preferentially between residues like cysteine, glycine, phenylalanine, valine, leucine and tyrosine (belonging to chain B). Besides being in agreement with a previous report (Cheng and Kawakishi, 1994), it is also in agreement with the results obtained with oxidized BSA samples with respect to peptide sequences that pointed out to be possible protein backbone cleavage sites occurring between residues such as tyrosine, leucine and glycine. These peptide sequences were likewise identified just in oxidized samples and with no typical tryptic cleavage sites.

In Paper 4, we studied the oxidative changes occurring in two histones, H2B and H1, by employing similar oxidant conditions and identifying the modifications through the same MS-based approach used so far. Our goal was to understand the oxidative behavior of these two very important proteins because of their *in vivo* biological role. In addition, this characterization represented an important task in spite of the differences in structural conformation and amino acid composition between the previous studied proteins and histones (for instance, histones do not possess cysteine residues nor cysteine disulfide bridges).

When histone H2B were submitted to *in vitro* oxidation, it showed to be affected mainly at methionine, phenylalanine, proline, lysine, histidine, glycine, asparagines and aspartic acid residues. With the exception of asparagine and aspartic acid, the remaining residues are known to be highly susceptible to lioxidation by MCO systems (Stadtman and Berlett, 1997; Stadtman and Levine, 2003; Guan and Chance, 2005) and were also found in oxidized BSA and insulin samples. Nevertheless, in the case of histone H2B residues like phenylalanine, glycine, histidine, asparagine and aspartic acid appeared oxidatively modified at earlier stages of oxidation even in milder conditions. This is likely related to the native structural conformation of the protein which allows an increased access to oxidant species. In fact, H2B oxidative damage initiated at the middle region of the protein where the highly susceptible methionine

residues are located. Further damage progressed to the end terminals of the protein as also observed previously for insulin.

With respect to oxidized histone H1, the major affected residues were proline, glycine, lysine and valine residues. Despite its particular amino acid composition as a lysine-rich histone, thus having more polar regions, H1 oxidation did not progress faster than the other studied proteins. As expected, we observed a significant oxidation of lysine residues. The remaining major affected residues were found to be located near identified oxidized lysines mainly in the end terminals of H1. Of particular notice was the identification of more products resulting from oxidation beyond the initial oxidative event (hydroxylation). Examples of these oxidation products are the oxidative conversion of lysine residues to aminoadipic derivatives, proline residues to pyroglutamic acid and glutamic semialdehyde, pyrrolidinone and pyrrolidone, oxidation of arginine residues to glutamic semialdehyde, and carbamylation of lysine residues. Similar observations were just seen for oxidized BSA samples but at longer oxidation periods and/or more aggressive oxidant conditions, with the identification of aminoadipic derivatives, histidine conversion to aspargine and aspartic acid, carbamylation of lysine and cysteine residues, proline oxidation to pyroglutamic acid and pyrrolidinone.

5.2 Identification of non-enzymatic glycation sites by mass spectrometry

The use of mass spectrometry in the identification and relative quantification of non-enzymatic glycated proteins has been well validated through reports available in the literature and revised in the introductory section of this dissertation. Mass spectrometry has been successfully applied in the study of early and advanced stages of glycation using MALDI and ESI for both qualitative and semi-quantitative analyses of glycated peptide mixtures (Lapolla *et al.*, 2001). This is accomplished by the identification of the number of hexose residues coupled to a given protein or peptide which is estimated by the mass increase of 162 Da per attached monosaccharide (Lapolla *et al.*, 2006).

Nevertheless, such analyses are seriously complicated by the presence of signals corresponding to other species such as cross-linked products. Also, a peptide mass fingerprint (PMF) is not sufficient to identify glycated peptides, as the mass shift of 162 Da is also observed for enzymatic O- and N-glycosylations (Mann and Jensen, 2003). So, tandem mass spectrometry has to be applied in order to obtain structural information (Lapolla *et al.*, 2004). To date, studies conducted on short N-terminally Amadori modified model peptides using ESI- and FAB-MS revealed characteristic fragmentation patterns (Jeric *et al.*, 2002; Horvat and Jakas, 2004). These patterns include consecutive losses of one (-18u) or three water molecules (-54u) and an additional molecule of formaldehyde (-84u) that yield oxonium, pyrylium, and furylium ions, respectively. This has allowed distinguishing glucose-derived N-terminally Amadori modified peptides from glycopeptides esters, cyclic Amadori compounds and peptide glycosylamines (Jeric *et al.*, 2002).

Based on the advantages of the use of MS in the characterization of protein glycation sites, we were able to successfully study the glycation of insulin and histones H2B and H1 (Paper 2 and Paper 4). The methodology used was similar to the one used for the analysis of the modifications following *in vitro* oxidative stress, involving HPLC separation of enzymatic digested peptides coupled to tandem mass spectrometry (MALDI-TOF/TOF) for the unambiguous assignment of the glycation sites. The interpretation of the tandem mass spectra took into consideration the reports by Frolov et al. (Frolov *et al.*, 2006; Frolov and Hoffmann, 2008), where D-glucose peptides studied by ESI- and MALDI-MS/MS displayed characteristic fragmentation patterns accompanying both parent and fragment ions, which could be explained by consecutive losses of water and formaldehyde as already reported (Jeric *et al.*, 2002), apart from the loss of the entire sugar moiety (-162Da) and the loss of part of the sugar moiety (-120Da) (Frolov and Hoffmann, 2008). So, each glycation site displayed a specific fragmentation of the sugar moiety, which was suitable to identify glycated *b*- and *y*-ions as well as to identify the glycation site within the peptide sequence.

In the case of the insulin glycation study (Paper 2), we were able to characterize for the first time three glycation sites when glycation of insulin was performed under reducing conditions as reported in the literature (O'Harte *et*

al., 1996; McKillop *et al.*, 2003; Farah *et al.*, 2005). In addition, two possible glycation sites were identified in insulin glycated under pseudophysiological conditions (in the absence of reducing agent). Our study also pointed out to the relevance of a specific glycation site, Lys-29, rather than solely the N-terminals of both chains (Gly-1 and Phe-1) as previously reported (O'Harte *et al.*, 2000).

The identification of the glycation sites in histones H2B and H1 (Paper 4) was reported for the first time as well. This was accomplished by digesting native and glycated samples with suitable enzymes such as endoproteinase Glu-C for histone H2B and endoproteinase Arg-C for histone H1. Resultant peptides were analyzed by nano-HPLC-MALDI-TOF/TOF likewise BSA and insulin.

In concurrence with the reports by Frolov et al. (Frolov *et al.*, 2006; Frolov and Hoffmann, 2008), we observed the fragmentation patterns of the glycated peptides were independent of the peptide sequence which infers this strategy as a general application in identifying glycation sites in proteins.

5.3 Mutual interaction between protein non-enzymatic glycation and oxidation

As reviewed in the introductory section (1.2.3), there is nowadays the term coined 'glycoxidation' which is recognized as the synergistic interaction between glycation and oxidative processes. These processes comprise the autoxidation of protein-bound sugars and the autoxidative glycosylation, in which radicals generated from free sugars interact with proteins (Wolff and Dean, 1987; Morgan *et al.*, 2002). Because the cumulative effect between glycation and oxidation events can represent one of the major responsible for the accumulation of non-functional damaged proteins, we intended to give evidence to the idea that such cumulative effect likely starts at the molecular level. A study by Traverso and co-workers (Traverso *et al.*, 1997) proposed the occurrence of molecular interactions between glycation and oxidation, with oxidized proteins being structurally more susceptible to glycation leading to enhanced advanced glycation modifications. By taking advantage of the high

potential of the MS-based approach employed in characterizing protein oxidative damage, we carried on studying the oxidative behavior of proteins previously submitted to glycation. We initially performed the study in insulin (Paper 3) which, besides being a smaller protein, only possesses a maximum of three possible glycation sites. On the opposite, histones H2B and H1 can form a larger number of glucose adducts, especially histone H1, allowing us to understand the influence of glycation with a more severe modified protein (Paper 4).

With respect to insulin and the characterization of its oxidative behavior when pre-glycated, along with the damage already identified in oxidized native insulin, we identified a higher number of oxidized residues that could be related to the presence of the glucose adducts. In addition, although insulin oxidative cleavage occurred mainly in the vicinity of the same type of residues as for oxidized native insulin, a higher number of fragments was detected for pre-glycated oxidized samples. As the number of glycation sites increased, the cumulative damage to insulin structure became more unambiguous. Both diglycated and triglycated insulin were the forms of insulin that showed the highest oxidative damage determined through the higher number of cleavage sites and oxidatively modified residues taking place throughout all structure. In B-chain, more fragments are formed not only in the vicinity of the residues found in less glycated insulin samples, but also in residues such as serine, asparagine and glutamine. Besides being identified more modified residues, we detected only in diglycated and triglycated samples, the formation of carbonyl derivatives in residues other than proline such as glycine, leucine and phenylalanine. Only in these samples was oxidative damage observed in A-chain, which was related to the other glycation site at Gly-1 of A-chain. Results showed the oxidation of residues located in close proximity with other modified residues from B-chain, suggesting this outcome as a consequence of further propagation of oxidative damage occurring solely in pre-glycated samples.

With respect to histones H2B and H1, our data pointed out to very similar results showing these two proteins were markedly more susceptible to oxidation when glycated. In agreement with insulin, we detected not only a higher number of oxidatively modified residues in pre-glycated samples but also its occurrence in an earlier stage when comparing with the results obtained for

oxidized native histones. Of notice is as well the higher occurrence of further oxidative modifications in residues such as lysine, proline, arginine and glycine that evolve from the initial hydroxyl derivative formation.

In spite of this concurrence in our data, we proposed this outcome as a consequence of alterations in protein conformation due to glycation, making possible and/or easing the access of oxidant species to protein zones otherwise inaccessible. These reactive species appear from the autoxidation of either glucose or Amadori intermediates, favored by the presence of metal ions (Argirova and Ortwerth, 2003), and diffuse to neighboring susceptible amino acid chains (Chetyrkin *et al.*, 2008). Moreover, there is evidence of AGE/CML-peptides binding redox-active transition metal ions and providing favorable conditions for hydroxyl radical formation, promoting the oxidation of nearby protein residues (Saxena *et al.*, 1999). Our results come in agreement since we observed that along the time-course of oxidation, damage occurred preferentially or initiated in the vicinity of glycated residues.

Altogether, our data contribute to the understanding at the molecular level that glycated proteins display a pro-oxidative behavior that, under the appropriate conditions, can promote the accumulation of non-functional damaged proteins that in turn enhances oxidative stress at the cellular level.

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6. Conclusions

After the first introduction of the concept of "proteome" more than 10 years ago, large-scale studies of protein expression, localization, activities and interactions have gained an exponential increase of interest, leading to extensive research and technology development. Proteomics approaches have been developed for protein separation and identification, and for the characterization of protein function and structure. Within these aims, characterization of protein posttranslational modifications have evolved as a great focus of research and, although challenging, it aspires to provide invaluable insights into cellular functions underlying biological processes both in normal and pathological conditions. Mass spectrometry approaches are of special value in the identification, characterization and quantification owing to issues such as femtomolar sensitivity of detection and simultaneous identification of peptide fragments bearing a particular type of chemical modification.

Among the most studied posttranslational modifications are non-enzymatic glycation and protein oxidation due to the evidences that account for glycation and oxidation as two important processes playing an important role in the complications of pathophysiological processes, such as biological aging and diabetes. Despite the studies focusing on the damage caused by these two events, there is no report on the effects of glycoxidative damage at the protein structure level. Therefore, our aim was to take advantage of proteomics for the study of oxidation and non-enzymatic glycation modifications in model systems. Our methodology involved the *in vitro* induction of protein oxidative stress and glycation, followed by liquid chromatography separation of the enzymatically digested peptides, and mass spectrometry analysis (MS and MS/MS) for the identification of peptide modifications and assignment of the modification location.

In all of the studied proteins, we observed that oxidative damage promoted the appearance of hydroxyl derivatives and carbonyl groups as the main oxidative modifications. Nevertheless, other oxidative modifications were detected such as deamidations, carbamylations and specific oxidative conversions on amino acids for instance histidine, arginine, proline and lysine. The time-course investigation enabled to give evidence that oxidative damage affects particular regions of the protein at different time points. Our results

indicated the oxidation initiates in surface-exposed regions (such as end terminals) and, if it is the case, near cysteine disulfide bridges or methionine residues. At this point, extremely susceptible amino acids like cysteine, methionine and tryptophan appear oxidized. Although not as susceptible as the cited residues, lysine and arginine can also appear modified in early stages of oxidation when present in exposed locations. As the damage propagates, neighboring regions comprising other susceptible but usually more shielded amino acids like phenylalanine, tyrosine, proline and histidine appear oxidized. If oxidation conditions were harsher, other less susceptible residues like glycine, leucine, valine and aspartic acid were eventually affected. Of notice was the number of oxidized residues not increasing linearly along the time course of the oxidation, which indicated that damage progression occurred dependently of the loss of stability in protein conformation. This was particularly observed for albumin where stronger oxidant conditions were used.

With oxidation following pre-glycation, all of the studied proteins showed to be more susceptible with the oxidative damage occurring earlier and more pronouncedly. Along with the similar damage observed for the corresponding native proteins, a higher number of newly oxidized amino acids were identified and higher percentage of modifications other than the primary hydroxylation event prevailed in oxidized pre-glycated proteins. These events were correlated with the location of the identified glycation sites within the protein sequence. Our results pointed to an increased oxidation-mediated in the vicinity of glycated residues. In conclusion, our data represent a contribution to the confirmation of glycated proteins as pro-oxidants and thus mediators in generating oxidative stress.

7. Appendix

Paper 1 supplemental table

Sample	Modification	Sequence	Position	Observed Mass	Calculated Mass	Match Error PPM	Match Error Da
BSA_c	Cys->Oxoalanine (C)[1,2,12], Dioxidation (P)[5]	CCTKPESERMPCTEDYLSLILNR	460-482	2679,5140	2679,2568	96	0,2572
	Lys->Allysine (K)[2,7]	LKECCDKPLLEK	298-309	1416,4557	1416,6749	-155	-0,2192
	Lys->Allysine (K)[12,15]	CVLHEKTPVSEKVTKCCTESLVNR	484-507	2700,9963	2701,2993	-112	-0,3030
	Oxidation (P)[8] or Oxidation (R)[15]	KVPQVSTPTLVEVSR	437-451	1655,7896	1655,9326	-86	-0,1430
30' Ox_2							
	Arg->GluSA (R)[13]	DAFLGSFLYEYSR	347-359	1524,5622	1524,5891	-18	-0,0269
	Carbamyl (C)[6], Carbamyl (M)[2]	DMADCCEKQEPERNECFLSH	110-129	2469,9106	2469,9526	-17	-0,0420
	Deamidated (R)[11]	HPEYAVSVLLR	361-371	1284,7048	1284,6947	8	0,0101
	Dioxidation (C)[7]	SQYLQQCPFDEHVK	52-65	1752,7761	1752,7777		-0,0016
	Dioxidation (P)[8,12]	KDDSPDLPKLKP	130-141	1415,7230	1415,7144		0,0086
	Dioxidation (Y)[2]	DYLSLILNRLCVL	475-486	1565,8399	1565,8487	-6	-0,0088
	Lys->Allysine (K)[14]	PYFYAPELLYYANKYN	170-185	2026,8983	2026,9352	-18	-0,0369
	Lys->Allysine (K)[12]	FYAPELLYYANK	172-183	1490,7510	1490,7202	21	0,0308
	Lys->AminoadipicAcid (K)[1]	KVPQVSTPTLVEVSR	437-451	1654,9131	1654,9010	7	0,0121
	Lys->Allysine (K)[16]	RPCFSALTPDETYVPK	508-523	1822,8984	1822,8680	17	0,0304

	Oxidation (Y)[1]	YLYEIAR	161-167	943,5093	943,4883	22	0,0210
	Oxidation (Y)[3]	YLYEIAR	161-167	943,5018	943,4883	14	0,0135
	Oxidation (Y)[9]	DAFLGSFLYEYSR	347-359	1583,5617	1583,5748	-8	-0,0131
	Oxidation (P)[8]	KVPQVSTPTLVEVSR	437-451	1655,9135	1655,9135	-12	-0,0191
	Oxidation (M)[1]	MPCTEDYLSLILNR	469-482	1682,8162	1682,8008	9	0,0154
	Oxidation (H)[8] or Oxidation (K)[11]	ALVELLKHKPKATEEQ	551-566	1849,7997	1849,8431	-23	-0,0434
	Pro->Pyrrolidinone (P)[2]	MPCTEDYLSLILNR	469-482	1637,8210	1637,8026	11	0,0184
	Quinone (Y)[1]	YNGVFQECCQAEDK	184-197	1663,6022	1663,6022	-20	-0,0340
	Trioxidation (C)[7]	SQYLQQCPFDEHVK	52-65	1769,7893	1769,7799	5	0,0094
	Trp->Oxolactone (W)[7]	ADEKKFWG	152-159	994,4019	994,4208	-19	-0,0189
30'	Ox_10						
	Amino (Y)[1], Carbamyl (R)[15]	YGDMADCCEKQEPERNECFLSH	108-129	2660,9771	2661,0352	-22	-0,0581
	Amino (Y)[11]	DAFLGSFLYEYSRR	347-360	1738,7959	1738,8547	-34	-0,0588
	Amino (Y)[9]	DAFLGSFLYEYSR	347-359	1582,7448	1582,7537	-6	-0,0089
	Arg->GluSA (R)[9]	GSFLYEYSRR	351-360	1234,6099	1234,6066	3	0,0033
	Carbamyl (K)[6,8]	LVELLKHKP	552-560	1161,6805	1161,6870	-6	-0,0065
	Cys->Oxoalanine (C)[4], Dioxidation (P)[18]	KSHCIAEVEKDAIPENLP	309-326	2005,9521	2005,9956	-22	-0,0435
	Cys->Oxoalanine (C)[3,13], Deamidated (R)[10]	RCCTKPESERMPCT	459-472	1605,6991	1605,6997	0	-0,0006

Cys->Oxoalanine (C)[10], Dioxidation (P)[1]	PVSEKVTKCCTESLVNR	491-507	1906,9243	1906,9539	-16	-0,0296
Dioxidation (R)[5], Dioxidation (K)[10,24]	CADDRADLAKYICDNQDTISSKLK	276-299	2782,1321	2782,1587	-10	-0,0266
Dioxidation (C)[2]	LCVLHEK	483-489	873,4324	873,4498	-20	-0,0174
Lys->AminoadipicAcid (K)[3]	RFKDLGEEHFK	34-44	1420,6365	1420,6855	-34	-0,0490
Lys->Allysine (K)[21]	GLVLIAFSQYLQQCPFDEHVK	45-65	2434,2266	2434,2112	6	0,0154
Lys->Allysine (K)[16]	RHPYFYAPELLYYANK	168-183	2044,0393	2043,9963	21	0,0430
Lys->Allysine (K)[12]	FYAPELLYYANK	172-183	1490,7532	1490,7202	22	0,0330
Lys->Allysine (K)[3]	VHKECCHGDLLECADDR	264-280	1941,7939	1941,7888	3	0,0051
Lys->Allysine (K)[2,7]	LKECCDKPLLEK	298-309	1416,7240	1416,6749	35	0,0491
Lys->AminoadipicAcid (K)[2]	RKVPQVSTPTLVEVSR	436-451	1810,9712	1811,0021	-17	-0,0309
Oxidation (R)[1] or Oxidation (K)[3] or Oxidation (D)[4]	RFKDLGEEHFK	34-44	1421,6932	1421,7172	-17	-0,0240
Oxidation (D)[3] or Oxidation (H)[8] or Oxidation (F)[9] or Oxidation (K)[10]	FKDLGEEHFK	35-44	1265,6387	1265,6161	18	0,0226
Oxidation (C)[14], Pro->pyro-Glu (P)[15]	GLVLIAFSQYLQQCPFDEHVK	42-65	2465,1992	2465,2170	-7	-0,0178
Oxidation (K)[15,25], Oxidation (N)[3], Oxidation (P)[1]	PENLPPLTADFAEDKDVCKNYQEAKDA	322-348	3085,4525	3085,3943	19	0,0582
Oxidation (D)[1] or Oxidation (Y)[9]	DAFLGSFLYEYSR	347-359	1583,7157	1583,7377	-14	-0,0220
Oxidation (Y)[4]	LGEYGFQNALIVR	421-433	1495,8016	1495,7903	8	0,0113
Oxidation (K)[1]	KVPQVSTPTLVEVSR	437-451	1655,9298	1655,9326	-2	-0,0028
Oxidation (M)[8] or Oxidation (P)[9] or Oxidation (C)[10] or Oxidation (D)[13] or Oxidation (Y)[14]	TKPESERMPCTEDYLSLILNR	462-482	2511,2068	2511,2217	-6	-0,0149
Oxidation (H)[8] or Oxidation (K)[11]	ALVELLKHKPKATEEQ	551-562	1849,9885	1850,0382	-27	-0,0497

	Pro->pyro-Glu (P)[8]	RHPYFYAPELLYYANK	168-183	2058,9675	2059,0073	-19	-0,0398
	Pro->Pyrrolidinone (P)[3]	KVPQVSTPTLVEVSR	437-451	1609,8990	1609,9272	-18	-0,0282
	Pro->Pyrrolidinone (P)[7], Trioxidation (C)[8]	PESERMPCTEDYLSLILNR	464-482	2284,0190	2284,0583	-17	-0,0393
	Pro->Pyrrolidinone (P)[4], Trioxidation (C)[12,13]	EKTPVSEKVTKCCTESLVNR	488-507	2317,0623	2317,1011	-17	-0,0388
	Pro->pyro-Glu (P)[1]	PVSEKVTKCCTESLVNR	491-507	1906,9243	1906,9362	-6	-0,0119
	Pro->Pyrrolidinone (P)[1]	PVSEKVTKCCTESLVNR	491-507	1862,8793	1862,9463	-36	-0,0670
	Quinone (Y)[10]	GLVLIAFSQYLQQCPFDEHVK	45-65	2465,1992	2465,2170	-7	-0,0178
	Trioxidation (C)[7]	SQYLQQCPFDEHVK	52-65	1769,7684	1769,7799	-6	-0,0115
	Trioxidation (C)[8]	PESERMPCTEDYLSLILNR	464-482	2314,0842	2314,0842	7	0,0151
	Trp->Oxolactone (W)[7]	ADEKKFWG	151-159	994,4965	994,4628	34	0,0337

Sample	Modification	Sequence	Position	Observed Mass	Calculated Mass	Match Error PPM	Match Error Da
1h Ox_2							
	Carbamyl (C)[6], Carbamyl (M)[2]	DMADCCEKQEPERNECFLSH	110-129	2470,0138	2469,9526	25	0,0612
	Deamidated (R)[7]	YLYEIAR	161-167	927,5012	927,4702		0,0311
	Deamidated (R)[13]	LGEYGFQNALIVR	421-433	1480,7922	1480,7794	9	0,0128
	Deamidated (R)[14]	MPCTEDYLSLILNR	469-482	1668,8192	1668,7972	13	0,0220
	Dioxidation (Y)[9,11]	DAFLGSFLYEYSR	347-359	1631,6384	1631,6265	7	0,0119

	Lys->Allysine (K)[12]	FYAPELLYYANK	172-183	1490,7448	1490,7202	17	0,0246
	Lys->Allysine (K)[1]	KVPQVSTPTLVEVSR	437-451	1639,0320	1638,9061	77	0,1259
	Oxidation (Y)	PYFYAPELLYYANKYN	170-185	2043,9231	2043,9618	-19	-0,0387
	Oxidation (F)[7], Oxidation (Y)[9,11], Oxidation (R)[13]	DAFLGSFLYEYSR	347-359	1631,6384	1631,6265	7	0,0119
	Oxidation (R)[9]	TPTLVEVSR	443-451	1016,5128	1016,5502	-37	-0,0374
	Oxidation (M)[9], Oxidation (C)[1], Pro->pyro-Glu (P)[4,10]	CTKPESERMPCTEDYLSLILNR	461-482	2658,5930	2658,5566	14	0,0364
	Oxidation (M)[1], Oxidation (C)[3]	MPCTEDYLSLILNR	469-482	1699,7728	1699,8030	-18	-0,0302
	Oxidation (H)[8]	ALVELLKHKPKATEEQ	551-566	1849,9990	1850,0382	-21	-0,0392
	Pro->Pyrrolidinone (P)[6]	PQVSTPTLVEVSRSLG	439-454	1639,9265	1639,9014	15	0,0251
	Trp->Oxolactone (W)[7]	ADEKKFWG	152-159	994,4520	994,4628	-11	-0,0108
1h	Ox_10						
	Amino (Y)[13]	RHPYFYAPELLYYANK	168-183	2060,0195	2060,0388	-9	-0,0193
	Amino (Y)[11]	PYFYAPELLYYANKYN	170-185	2044,0009	2043,9851	8	0,0158
	Amino (Y)[1,2,6]	YYANKYNGVFQECCQAEDKGACL	179-201	2662,1077	2662,1482	-15	-0,0405
	Amino (Y)[11]	DAFLGSFLYEYSRR	347-360	1738,7974	1738,8547	-33	-0,0573
	Arg->GluSA (R)[13]	LGEYGFQNALIVR	421-433	1436,7476	1436,7476	4	0,0057
	Carbamyl (K)[1]	KLVNELTEFAK	65-75	1334,6975	1334,7314	-25	-0,0339
	Carbamyl (C)[4,5,12]	HKECCHGDLLECADDR	265-280	1972,7831	1972,7695	7	0,0136
	Carbamyl (K)[6]	NYQEAKDAFLGS	341-352	1385,6317	1385,6332	-1	-0,0015

Carbamyl (K)[1]	KDAFLGSFLYEYSR	346-359	1738,7974	1738,8435	-27	-0,0461
Carbamyl (K)[1]	KHLVDEPQNLIK	401-412	1476,7944	1476,8169	-15	-0,0225
Carbamyl (K)[1]	KLGEYGFQNALIVR	420-433	1650,8336	1650,8962	-38	-0,0626
Carbamyl (K)[1]	KVPQVSTPTLVEVSR	437-451	1682,9451	1682,9436	1	0,0015
Carbamyl (K)[26], Oxidation (H)[16]	EKQIKKQTALVELLKHKPKATEEQLKTVM	543-571	3449,0469	3448,9558	26	0,0911
Cys->Oxoalanine (C)[7], Dioxidation (P)[8]	SQYLQQCPFDEHVK	52-65	1735,8049	1735,7922	7	0,0127
Cys->Oxoalanine (C)[6,13]	VHKECCHGDLLECADDR	264-280	1906,8395	1906,8348	2	0,0047
Dioxidation (Y)[10]	GLVLIAFSQYLQQCPFDEHVK	45-65	2466,2077	2466,2253	-7	-0,0176
Dioxidation (Y)[3] or Dioxidation (C)[7]	SQYLQQCPFDEHVK	52-65	1753,7927	1753,7850	4	0,0077
Dioxidation (C)[5,12]	HKECCHGDLLECADDRADLAKY	265-286	2569,0955	2569,0752	8	0,0203
Dioxidation (M)[1]	MPCTEDYLSLILNR	469-482	1699,7104	1699,6740	21	0,0364
Dioxidation (Y)[2]	DYLSLILNRLCVL	474-486	1565,8099	1565,8487	-25	-0,0388
His->Asp (H)[19]	GLVLIAFSQYLQQCPFDEHVKLVNELT	45-71	3082,4535	3082,3936	19	0,0599
His->Asn (H)[12]	SQYLQQCPFDEHVKL	52-66	1811,8723	1811,8633	5	0,0090
Lys->AminoadipicAcid (K)[3]	RFKDLGEEHFK	34-44	1420,6708	1420,6855	-10	-0,0147
Lys->Allysine (K)[21]	GLVLIAFSQYLQQCPFDEHVK	45-65	2434,2466	2434,2112	15	0,0354
Lys->Allysine (K)[16]	RHPYFYAPELLYYANK	168-183	2043,9982	2043,9963	1	0,0019
Lys->AminoadipicAcid (K)[14]	PYFYAPELLYYANKYN	170-185	2043,9227	2043,9375	-7	-0,0148
Lys->Allysine (K)[12]	FYAPELLYYANK	172-183	1490,7522	1490,7202	21	0,0320
Lys->Allysine (K)[12]	YICDNQDTISSK	286-297	1385,6262	1385,5890	27	0,0372
Lys->Allysine (K)[2], Lys->AminoadipicAcid (K)[7], Oxidation (D)[6]	LKECCDKPLLEK	298-309	1448,7024	1448,6648	26	0,0376
Lys->Allysine (K)[9,13], Oxidation (D)[8]	LTADFAEDKDVCKN	328-341	1582,7004	1582,6578	27	0,0426
Lys->AminoadipicAcid (K)[1]	KDAFLGSFLYEYSR	346-359	1710,7689	1710,8009	-19	-0,0320

Lys->Allysine (K)[11]	HLVDEPQNLIK	402-412	1304,6942	1304,6846	7	0,0096
Lys->AminoadipicAcid (K)[2]	RKVPQVSTPTLVEVSR	436-451	1810,9761	1811,0021	-14	-0,0260
Lys->Allysine (K)[1]	KVPQVSTPTLVEVSR	437-451	1638,9091	1638,9061	2	0,0030
Lys->Allysine (K)[16]	RPCFSALTPDETYVPK	508-523	1822,8997	1822,8680	17	0,0317
Lys->Allysine (K)[12]	TVMENFVAFVDK	569-580	1398,6893	1398,6610	20	0,0283
Oxidation (F)[1] or Oxidation (K)[2] or Oxidation (D)[3] or Oxidation (H)[8]	FKDLGEEHFK	35-44	1265,6232	1265,6161	6	0,0071
Oxidation (C)[7], Pro->pyro-Glu (P)[8]	SQYLQQCPFDEHVK	52-65	1751,7766	1751,7694	4	0,0072
Oxidation (Y)[1]	YLYEIAR	161-167	943,5034	943,4883	16	0,0151
Oxidation (Y)[6] or Oxidation (P)[8]	RHPYFYAPELLYYANK	168-183	2061,0046	2061,0229	-9	-0,0183
Oxidation (K)[1]	KDAFLGSFLYEYSR	346-359	1711,7853	1711,8326	-28	-0,0473
Oxidation (D)[1] or Oxidation (F)[7] or Oxidation (Y)[9]	DAFLGSFLYEYSR	347-359	1583,7168	1583,7377	-13	-0,0209
Oxidation (H)[2] or Oxidation (P)[3] or Oxidation (Y)[5]	RHPEYAVSVLLR	360-371	1455,7886	1455,8066	-12	-0,0180
Oxidation (H)[1] or Oxidation (D)[4]	HLVDEPQNLIK	402-412	1321,7125	1321,7111	1	0,0014
Oxidation (Y)[4] or Oxidation (N)[8]	LGEYGFQNALIVR	421-433	1495,7722	1495,7903	-12	-0,0181
Oxidation (R)[1] or Oxidation (K)[2]	RKVPQVSTPTLVEVSR	436-451	1811,9961	1812,0338	-21	-0,0377
Oxidation (P)[8]	KVPQVSTPTLVEVSR	437-451	1655,9347	1655,9347	1	0,0021
Oxidation (M)[10]	CCTKPESERMPCTEDYLSLILNR	460-482	2717,0157	2717,0728	-21	-0,0571
Oxidation (M)[1], Oxidation (P)[2]	MPCTEDYLSLILNR	469-482	1683,8210	1683,8081	8	0,0129
Oxidation (M)[1], Oxidation (C)[3]	MPCTEDYLSLILNR	469-482	1699,8104	1699,8030	4	0,0074
Oxidation (C)[9], Pro->pyro-Glu (P)[12]	LFTFHADICTLPD	529-541	1521,6734	1521,6810		-0,0075
Pro->pyro-Glu (P)[15]	GLVLIAFSQYLQQCPFDEHVK	45-65	2449,2783	2449,2219	23	0,0564
Pro->pyro-Glu (P)[8]	SQYLQQCPFDEHVK	52-65	1735,8049	1735,7744	18	0,0305
Pro->pyro-Glu (P)[3]	RHPYFYAPELLYYANK	168-183	2058,9993	2059,0073	-4	-0,0080

	Pro->pyro-Glu (P)[3]	RHPEYAVSVLLR	360-371	1453,7925	1453,7910	1	0,0015
	Pro->Pyrrolidinone (P)[22], Trioxidation (C)[18,19]	TPTLVEVSRSLGKVGTRCCTKPESER	443-468	2898,3909	2898,4175	-9	-0,0266
	Pro->Pyrrolidinone (P)[1]	PVSEKVTKCCTESLVNR	491-507	1861,9310	1861,9390	-4	-0,0080
	Quinone (Y)[10]	GLVLIAFSQYLQQCPFDEHVK	45-65	2465,2107	2465,2170	-3	-0,0063
	Quinone (Y)[3]	SQYLQQCPFDEHVK	52-65	1751,7554	1751,7554	-8	-0,0140
	Trioxidation (C)[7]	SQYLQQCPFDEHVK	52-65	1768,7750	1768,7726		0,0024
	Trioxidation (C)[4,5]	HKECCHGDLLECADDR	265-280	1938,7300	1938,7142	8	0,0158
	Trioxidation (C)[8]	PESERMPCTEDYLSLILNR	464-482	2314,0139	2314,0691	-24	0,0552
	Trp->Oxolactone (W)[7]	ADEKKFWG	152-159	994,4273	994,4628	-36	-0,0355

Sample	Modification	Sequence	Position	Observed Mass	Calculated Mass	Match Error PPM	Match Error Da
2h Ox_2							
	Amino (Y)[13]	RHPYFYAPELLYYANK	168-183	2060,0308	2060,0388	-4	-0,0080
	Amino (Y)[11,15]	PYFYAPELLYYANKYN	170-185	2058,9934	2058,9958	-1	-0,0024
	Amino (Y)[1,2,6]	YYANKYNGVFQECCQAEDKGACL	179-201	2662,2033	2662,1482	21	0,0551
	Amino (Y)[9]	DAFLGSFLYEYSR	347-359	1582,7220	1582,7537	-20	-0,0317
	Arg->GluSA (R)[13]	DAFLGSFLYEYSR	347-359	1524,6510	1524,6893	-25	-0,0383
	Carbamyl (K)[1]	KDAFLGSFLYEYSR	346-359	1738,7818	1738,8435	-35	-0,0617
	Carbamyl (K)[1]	KHLVDEPQNLIK	401-412	1476,7904	1476,8169	-18	-0,0265

Cys->Oxoalanine (C)[7], Dioxidation (P)[8]	SQYLQQCPFDEHVK	52-65	1735,7845	1735,7922	-4	-0,0077
Dioxidation (C)[4]	LQQCPFDEHVK	55-65	1375,6377	1375,6311	5	0,0066
Dioxidation (C)[3]	YICDNQDTISSK	286-297	1418,5997	1418,6105	-8	-0,0108
Dioxidation (Y)[7] or Dioxidation (K)[10] or Dioxidation (P)[12]	NALIVRYTRKVP	428-439	1461,7980	1461,8536	-38	-0,0556
Dioxidation (Y)[2]	DYLSLILNRLCVL	474-486	1566,7927	1566,8560	-40	-0,0633
Dioxidation (C)[2]	CCTESLVNR	499-507	1056,4215	1056,4448	-22	-0,0233
Dioxidation (K)[1,2,11,13]	KKQTALVELLKHKPKAT	547-563	2060,1001	2060,1477	-23	-0,0476
His->Asp (H)[19]	GLVLIAFSQYLQQCPFDEHVKLVNELT	45-71	3082,5107	3082,5806	-23	-0,0699
His->Asn (H)[3], His->Asp (H)[28]	TFHADICTLPDTEKQIKKQTALVELLKHKPKATE	531-564	3830,1515	3830,0620	23	0,0895
Lys->AminoadipicAcid (K)[3]	RFKDLGEEHFK	34-44	1420,6554	1420,6855	-21	-0,0301
Lys->Allysine (K)[2]	FKDLGEEHFK	35-44	1248,6082	1248,5895	15	0,0187
Lys->Allysine (K)[21]	GLVLIAFSQYLQQCPFDEHVK	45-65	2434,1941	2434,2112	-7	-0,0171
Lys->AminoadipicAcid (K)[10], Oxidation (D)[3,6]	YGDMADCCEKQEPERNECF	108-126	2312,8299	2312,7966	14	0,0333
Lys->Allysine (K)[5,7,22,23], Lys->AminoadipicAcid (K)[18], Oxidation (D)[15,20]	PDLPKLKPDPNTLCDEFKADEKKF	134-157	2831,2336	2831,2493	-6	-0,0157
Lys->Allysine (K), 3 Lys->AminoadipicAcid (K), 2 Oxidation (D)	LKPDPNTLCDEFKADEKKFWGKYL	139-162	2959,2984	2959,2633	12	0,0351
Lys->Allysine (K)[16]	RHPYFYAPELLYYANK	168-183	2044,0449	2043,9963	24	0,0486
Lys->AminoadipicAcid (K)[16]	RHPYFYAPELLYYANK	168-183	2060,0308	2059,9912	19	0,0396
Lys->Allysine (K)[14]	PYFYAPELLYYANKYN	170-185	2027,9922	2027,9425	25	0,0497
Lys->Allysine (K)	ISSKLKECCDKPL	294-306	1461,7600	1461,7207	27	0,0393
Lys->Allysine (K)[2,7]	LKECCDKPLLEK	298-309	1416,7002	1416,6749	18	0,0253
Lys->Allysine (K)[11]	HLVDEPQNLIK	402-412	1304,7188	1304,6846	26	0,0342

Lys->Allysine (K)[1]	KVPQVSTPTLVEVSR	437-451	1638,9312	1638,9061	15	0,0251
Lys->Allysine (K)[16]	RPCFSALTPDETYVPK	508-523	1822,8662	1822,8680	-1	-0,0018
Lys->Allysine (K)[11]	ALVELLKHKPKATEEQ	551-566	1833,0128	1833,0116	1	0,0012
Lys->Allysine (K)[12,19]	TVMENFVAFVDKCCAADDKEACFAVEGPK	569-597	3134,3009	3134,3380	-12	-0,0371
Oxidation (F)[1] or Oxidation (D)[3] or Oxidation (H)[8] or Oxidation (K)[10]	FKDLGEEHFK	35-44	1265,6270	1265,6161	9	0,0109
Oxidation (Y)[3]	YLYEIAR	161-167	943,4936	943,4883	6	0,0053
Oxidation (Y)[22,24], Oxidation (R)[26,27]	EDKDVCKNYQEAKDAFLGSFLYEYSRR	334-360	3338,5673	3338,5269	12	0,0404
Oxidation (D)[1] or Oxidation (F)[3] or Oxidation (Y)[9]	DAFLGSFLYEYSR	347-359	1583,7327	1583,7377	-3	-0,0050
Oxidation (R)[1] or Oxidation (P)[3] or Oxidation (H)[2]	RHPEYAVSVLLR	360-371	1455,7964	1455,8066	-7	-0,0102
Oxidation (Y)[4] or Oxidation (N)[8]	LGEYGFQNALIVR	421-433	1495,7830	1495,7903	-5	-0,0073
Oxidation (Y)[7], Oxidation (R)[9] or Oxidation (K)[10], Oxidation (P)[12]	NALIVRYTRKVP	428-439	1461,7960	1461,8536	-39	-0,0576
Oxidation (P)[3]	KVPQVSTPTLVEVSR	437-451	1655,9342	1655,9326	1	0,0016
Oxidation (M)[6], Oxidation (C)[8], Pro->pyro-Glu (P)[1]	PESERMPCTEDYLSLILNR	464-482	2312,0854	2312,0532	14	0,0322
Oxidation (C)[1,2]	CCTESLVNR	499-507	1056,3973	1056,4448	-45	-0,0475
Oxidation (K)[11] or Oxidation (H)[8]	ALVELLKHKPKATEEQ	551-566	1849,9855	1850,0382	-28	-0,0527
Pro->Pyrrolidinone (P)[7], Trioxidation (C)[8]	PESERMPCTEDYLSLILNR	464-482	2284,0701	2284,0583	5	0,0118
Pro->Pyrrolidinone (P)[2]	MPCTEDYLSLILNR	469-482	1637,7820	1637,8026	-13	-0,0206
Pro->Pyrrolidinone (P)[1]	PVSEKVTKCCTESLVNR	491-507	1862,8879	1862,9463	-31	-0,0584
Quinone (Y)[15]	PYFYAPELLYYANKYN	170-185	2059,0293	2058,9482	39	0,0811

	Quinone (Y)	YNGVFQECCQAEDK	184-197	1662,6092	1662,6290	-12	-0,0198
	Quinone (Y)[33]	FAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYA	332-365	4119,7876	4119,8818	-23	-0,0942
	Quinone (Y)[8]	QNALIVRYTRKVPQVSTPTL	427-446	2314,1945	2314,2878	-40	-0,0933
	Trioxidation (C)[7]	SQYLQQCPFDEHVK	52-65	1769,7677	1769,7799	-7	-0,0122
	Trioxidation (C)[8]	GKVGTRCCTKPESER	454-468	1698,7604	1698,7898	-17	-0,0294
	Trp->Oxolactone (W)[7]	ADEKKFWG	152-159	994,4590	994,4628	-4	-0,0038

Sample	Modification	Sequence	Position	Observed Mass	Calculated Mass	Match Error PPM	Match Error Da
2h Ox_10							
	Amino (Y)[13]	RHPYFYAPELLYYANK	168-183	2060,0403	2060,0388	1	0,0015
	Amino (Y)[1,2,6]	YYANKYNGVFQECCQAEDKGACL	179-201	2662,2533	2662,1482	39	0,1051
	Amino (Y)[9]	DAFLGSFLYEYSR	347-359	1582,7451	1582,7537	-5	-0,0086
	Amino (Y)[6,18], Arg->GluSA (R)[2,13]	RRHPEYAVSVLLRLAKEYEATLEE	359-382	2816,3568	2816,4465	-32	-0,0897
	Arg->GluSA (R)[13]	DAFLGSFLYEYSR	347-359	1524,6163	1524,5601	37	0,0562
	Carbamyl (K)[3]	ALKAWSVAR	233-241	1044,6163	1044,5948	21	0,0215
	Carbamyl (K)[5,8] or Carbamyl (C)[11,18]	TDLTKVHKECCHGDLLECADDR	259-280	2587,0944	2587,1333	-15	-0,0389
	Carbamyl (K)[1]	KHLVDEPQNLIK	401-412	1476,7599	1476,8169	-39	-0,0570
	Carbamyl (K)[1]	KVPQVSTPTLVEVSR	437-451	1682,9283	1682,9436	-9	-0,0153
	Cys->Oxoalanine (C)[3]	MPCTEDYLSLILNR	469-482	1649,8226	1649,8203	1	0,0023
	Cys->Oxoalanine (C)[9], Dioxidation (P)[1]	PVSEKVTKCCTESLVNR	491-507	1906,9208	1906,9539	-17	-0,0331

Cys->Oxoalanine (C)[3], Deamidated (R)[10,11], Dioxidation (P)[12]	KCCTESLVNRRPCFSALT	498-515	2043,9184	2043,9475	-14	-0,0291
Deamidated (R)	PTLVEVSRSLGKVGTRCCTKPESER	444-468	2732,4399	2732,3949	16	0,0450
Dioxidation (Y)[10]	GLVLIAFSQYLQQCPFDEHVK	45-65	2467,2595	2467,2327	11	0,0268
Dioxidation (C)[14] or Dioxidation (P)[15]	GLVLIAFSQYLQQCPFDEHVK	45-65	2467,2670	2467,2327	14	0,0343
Dioxidation (C)[4]	LQQCPFDEHVK	55-65	1375,6228	1375,6311	-6	-0,0083
Dioxidation (R)[9], Dioxidation (K)[4]	LLPKIETMR	201-209	1163,5792	1163,6220	-37	-0,0428
Dioxidation (Y)[2]	DYLSLILNRLCVL	474-486	1565,8078	1565,8487	-26	-0,0409
Dioxidation (C)[2]	CCTESLVNR	499-507	1056,4316	1056,4448	-12	-0,0132
His->Asp (H)[19]	GLVLIAFSQYLQQCPFDEHVKLVNELT	45-71	3082,6343	3082,5806	17	0,0537
Lys->AminoadipicAcid (K)[3]	RFKDLGEEHFK	34-44	1420,6753	1420,6855	-7	-0,0102
Lys->Allysine (K)[2]	FKDLGEEHFK	35-44	1248,6136	1248,5895	19	0,0241
Lys->Allysine (K)[21]	GLVLIAFSQYLQQCPFDEHVK	45-65	2434,2344	2434,2112	10	0,0232
Lys->Allysine (K)[16]	RHPYFYAPELLYYANK	168-183	2044,0024	2043,9963	3	0,0061
Lys->Allysine (K)[12]	FYAPELLYYANK	172-183	1490,7499	1490,7202	20	0,0297
Lys->Allysine (K)[2,7]	LKECCDKPLLEK	298-309	1416,7101	1416,6749	25	0,0352
Lys->Allysine (K)[2], Lys->AminoadipicAcid (K)[7], Oxidation (D)[6]	LKECCDKPLLEK	298-309	1448,6895	1448,6648	17	0,0247
Lys->AminoadipicAcid (K)[7], Oxidation (D)[2,6,8]	ADFAEDKDV	330-338	1071,4120	1071,3880	22	0,0240
Lys->AminoadipicAcid (K)[1]	KDAFLGSFLYEYSR	346-359	1710,7836	1710,8009	-10	-0,0173
Lys->Allysine (K)[1]	KVPQVSTPTLVEVSR	437-451	1638,9266	1638,9061	13	0,0205
Lys->Allysine (K)[12,19]	TVMENFVAFVDKCCAADDKEACFAVEGPK	569-597	3135,3716	3135,3455	8	0,0261

Oxidation (R)[1] or Oxidation (K)[3] or Oxidation (D)[4]	RFKDLGEEHFK	34-44	1421,6733	1421,7172	-31	-0,0439
Oxidation (F)[1] or Oxidation (K)[2] or Oxidation (D)[3] or Oxidation (H)[8]	FKDLGEEHFK	35-44	1265,6274	1265,6161	9	0,0113
Oxidation (F)[16], Oxidation (Y)[10]	GLVLIAFSQYLQQCPFDEHVK	45-65	2467,2678	2467,2327	14	0,0351
Oxidation (F)[7], Oxidation (Y)[10]	GLVLIAFSQYLQQCPFDEHVK	45-65	2467,2595	2467,2327	11	0,0268
Oxidation (C)[7], Pro->pyro-Glu (P)[8]	SQYLQQCPFDEHVK	52-65	1751,7538	1751,7694	-9	-0,0156
Oxidation (F)[8] or Oxidation (K)[10]	LVNELTEFAK	66-75	1179,6459	1179,6256	17	0,0203
Oxidation (Y)[1]	YLYEIAR	161-167	943,5011	943,4883	14	0,0128
Oxidation (P)[8] or Oxidation (Y)[13]	RHPYFYAPELLYYANK	168-183	2061,0061	2061,0229	-8	-0,0168
Oxidation (K)[3,11]	FPKAEFVEVTKLVTD	246-260	1754,8697	1754,9210	-29	-0,0513
Oxidation (F)[23]	SHCIAEVEKDAIPENLPPLTADFAEDKDV	310-338	3181,6006	3181,5125	28	0,0881
Oxidation (D)[1] or Oxidation (Y)[9]	DAFLGSFLYEYSR	347-359	1583,7025	1583,7377	-22	-0,0352
Oxidation (H)[2] or Oxidation (P)[3]	RHPEYAVSVLLR	360-371	1455,7991	1455,8066	-5	-0,0075
Oxidation (Y)[4] or Oxidation (N)[8]	LGEYGFQNALIVR	421-433	1495,8027	1495,7903	8	0,0124
Oxidation (K)[1]	KVPQVSTPTLVEVSR	437-451	1655,9231	1655,9326	-6	-0,0095
Oxidation (M)[1] or Oxidation (P)[2]	MPCTEDYLSLILNR	469-482	1683,7905	1683,8081	-10	-0,0176
Oxidation (K)[8] or Oxidation (C)[10]	PVSEKVTKCCTESLVNR	491-507	1908,9187	1908,9518	-17	-0,0331
Oxidation (C)[1,2]	CCTESLVNR	499-507	1056,4316	1056,4448	-12	-0,0132
Oxidation (K)[11]	ALVELLKHKPKATEEQ	551-566	1849,9592	1850,0382	-43	-0,0790
Pro->pyro-Glu (P)[6,10,12]	DSPDLPKLKPDPN	132-144	1477,7077	1477,6805	18	0,0272
Pro->pyro-Glu (P)[8]	RHPYFYAPELLYYANK	168-183	2058,9812	2059,0073	-13	-0,0261
Pro->pyro-Glu (P)[3]	RHPEYAVSVLLR	360-371	1453,7777	1453,7910	-9	-0,0133
Pro->Pyrrolidinone (P)[7], Trioxidation (C)[8]	PESERMPCTEDYLSLILNR	464-482	2284,0457	2284,0583	-6	-0,0126
Pro->Pyrrolidone (P)[2]	MPCTEDYLSLILNR	469-482	1639,8211	1639,8182	2	0,0029
Pro->pyro-Glu (P)[1]	PVSEKVTKCCTESLVNR	491-507	1906,9208	1906,9362	-8	-0,0154
Pro->Pyrrolidinone (P)[1]	PVSEKVTKCCTESLVNR	491-507	1862,9907	1862,9463	24	0,0444

Quinone (Y)[3]	SQYLQQCPFDEHVK	52-65	1751,7538	1751,7538	-9	-0,0156
Quinone (Y)[1]	YNGVFQECCQAEDK	184-197	1663,6180	1663,6362	-11	-0,0182
Trp->Oxolactone (W)[7]	ADEKKFWG	152-159	994,3819	994,4011	-19	-0,0192

Paper 3 supplemental table

Sample	Sequence	Position	Observed Mass	Calculated Mass	Match Error Da	Match Error PPM	Modification	Glu-C digests
Native								
4h								
	GERGFFYTPKA	B 20-30	1272,6410	1272,6371	0,0039	3		
	GERGFFYTPKA	B 20-30	1288,6188	1288,6321	-0,0133	-10	Oxidation (Y)[7]	
	GIVEQCCASVCSLYQLENYCN	A 1-21	2336,9509	2336,9660	-0,0151	-6		
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3398,6772	3398,6819	-0,0047	-1		
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5730,6988	5730,6089	0,0899	16		
24h								
	SHLVEAL	B 9-15	768,4325	768,4255	0,0070	9		
	RGFFYTPKA	B 22-30	1086,5977	1086,5731	0,0246	23		*
	ERGFFYTPKA	B 21-30	1215,6451	1215,6157	0,0294	24		*
	ERGFFYTPKA	B 21-30	1231,6273	1231,6106	0,0167	14	Oxidation (F)[5]	*
	GERGFFYTPKA	B 20-30	1272,6081	1272,6371	-0,0290	-23		
	GERGFFYTPKA	B 20-30	1288,6639	1288,6321	0,0318	25	Oxidation (F)[6]	
	GIVEQCCASVCSLYQLENYCN	A 1-21	2334,8442	2334,9504	-0,1062	-45		
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3396,4109	3398,6819	-2,2710			
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5730,1748	5730,6089	-0,4341	-76		

48h

SHLVEAL	B 9-15	768,4376	768,4255	0,0121	16	
FFYTPKA	B 24-30	873,4616	873,4505	0,0111	13	
GFFYTPKA	B 23-30	930,4919	930,4720	0,0199	21	
RGFFYTPKA	B 22-30	1086,5905	1086,5731	0,0174	16	*
RGFFYTPKA	B 22-30	1102,5597	1102,5680	-0,0083	-8	Oxidation (Y)[5] *
VEALYLVCGE	B 12-21	1143,5486	1143,5238	0,0248	22	Cysteic acid (C)[8] *
LYLVCGERGF	B 15-24	1156,6030	1156,5819	0,0211	18	*
LYLVCGERGF	B 15-24	1172,5774	1172,5769	0,0005	0	Oxidation (Y)[2] *
ERGFFYTPKA	B 21-30	1215,6344	1215,6157	0,0187	15	*
ERGFFYTPKA	B 21-30	1231,6094	1231,6106	-0,0012	-1	Oxidation (F)[4] *
ALYLVCGERGF	B 14-24	1243,6345	1243,6140	0,0205	16	Oxidation (Y)[3] *
ERGFFYTPKA	B 21-30	1247,6011	1247,6127	-0,0116	-9	Oxidation (F)[5], Oxidation (Y)[6] *
ALYLVCGERGF	B 14-24	1259,6288	1259,6089	0,0199	16	Oxidation (Y)[3], Oxidation (F)[11] *
GERGFFYTPKA	B 20-30	1272,6547	1272,6371	0,0176	14	
GERGFFYTPKA	B 20-30	1288,6289	1288,6321	-0,0032	-2	Oxidation (F)[5]
LYLVCGERGFF	B 15-25	1367,6512	1367,6300	0,0212	16	Oxidation (Y)[2], Cysteic acid (C)[5] *
GIVEQCCASVCSLYQLENYCN	A 1-21	2338,9346	2338,9816	-0,0470	-20	(no intrachain disulfide bridge)
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3398,6887	3398,6819	0,0068		

Sample	Sequence	Position	Observed Mass	Calculated Mass	Match Error Da	Match Error PPM	Modification	Glu-C digests
NRed1g								

4h

RGFFYTPKA	B 22-30	1086,5537	1086,5731	-0,0194	-18	*
ERGFFYTPKA	B 21-30	1215,6085	1215,6157	-0,0072	-6	*
ERGFFYTPKA	B 21-30	1231,5897	1231,6106	-0,0209	-17	Oxidation (Y)[6] *
GERGFFYTPKA	B 20-30	1272,6375	1272,6371	0,0004	0	
GERGFFYTPKA	B 20-30	1288,6430	1288,6321	0,0109	8	Oxidation (Y)[7]
GIVEQCCASVCSLYQLENYCN	A 1-21	2336,9871	2336,9660	0,0211	9	
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3560,7678	3560,7347	0,0331	9	Glycation (N)[3] or Glycation (K)[29]
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3576,7939	3576,7296	0,0643	18	Glycation (N)[3] or Glycation (K)[29], Oxidation
GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5892,9150	5892,6617	0,2533	43	Glycation (N)[3] or Glycation (K)[29]

24h

GFFYTPKA	B 23-30	930,4513	930,4720	-0,0207	-22	
RGFFYTPKA	B 22-30	1086,5728	1086,5731	-0,0003	0	*
RGFFYTPKA	B 22-30	1102,5513	1102,5680	-0,0167	-15	Oxidation (F)[4] or Oxidation (Y)[5] *
ERGFFYTPKA	B 21-30	1215,5923	1215,6157	-0,0234	-19	*
ERGFFYTPKA	B 21-30	1231,5885	1231,6106	-0,0221	-18	Oxidation (F)[5] or Oxidation (Y)[6] *
GERGFFYTPKA	B 20-30	1272,6066	1272,6371	-0,0305	-24	
GERGFFYTPKA	B 20-30	1288,6196	1288,6321	-0,0125	-10	Oxidation (F)[5 or 6]
GIVEQCCASVCSLYQLENYCN	A 1-21	2338,9748	2338,9816	-0,0068	-3	(no intrachain disulfide bridge)
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3560,7614	3560,7347	0,0267	7	Glycation (F)[1] or Glycation (K)[29]
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3576,7177	3576,7296	-0,0119	-3	Glycation (F)[1] or Glycation (K)[29], Oxidation

	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3592,7553	3592,7245	0,0308	9	Glycation (F)[1] or Glycation (K)[29], Dioxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5892,9082	5892,6617	0,2465	42	Glycation (F)[1] or Glycation (K)[29]	
48h								
	SHLVEAL	B 9-15	768,4381	768,4255	0,0126	16		
	GFFYTPKA	B 23-30	930,4887	930,4720	0,0167	18		
	RGFFYTPKA	B 22-30	1086,5847	1086,5731	0,0116	11		*
	RGFFYTPKA	B 22-30	1102,5781	1102,5680	0,0101	9	Oxidation (F)[4]	*
	ERGFFYTPKA	B 21-30	1215,6273	1215,6157	0,0116	10		*
	ERGFFYTPKA	B 21-30	1231,6155	1231,6106	0,0049	4	Oxidation (F)[4]	*
	ALYLVCGERGF	B 14-24	1243,6317	1243,6140	0,0177	14	Oxidation (Y)[3]	*
	ERGFFYTPKA	B 21-30	1247,6010	1247,6127	-0,0117	-9	Oxidation (F)[5], Oxidation (Y)[6]	*
	RGFFYTPKA	B 22-30	1248,6349	1248,6264	0,0085	7	Glycation (K)[8]	*
	ALYLVCGERGF	B 14-24	1259,6217	1259,6089	0,0128	10	Oxidation (Y)[3], Oxidation (F)[11]	*
	GERGFFYTPKA	B 20-30	1272,6586	1272,6371	0,0215	17		
	GERGFFYTPKA	B 20-30	1288,6441	1288,6321	0,0120	9	Oxidation (F)[5 or 6]	
	SHLVEALYLVC	B 9-19	1342,6448	1342,6451	-0,0003	0	Oxidation (H)[2], Dioxidation (Y)[8], Cysteic acid (C)[11]	
	CGERGFFYTPKA	B 19-30	1375,6392	1375,6469	-0,0077	-6		
	VCGERGFFYTPKA	B 18-30	1636,7318	1636,7681	-0,0363	-22	Glycation (K)[12]	
	VCGERGFFYTPKA	B 18-30	1652,7360	1652,7630	-0,0270	-16	Glycation (K)[12], Oxidation (F)[7 or 8]	

Sample	Sequence	Position	Observed Mass	Calculated Mass	Match Error Da	Match Error PPM	Modification	Glu-C digests
Red1g								
4h								
	ERGFFYTPKA	B 21-30	1231,6066	1231,6106	-0,0040	-3	Oxidation (F)[4] or Oxidation (Y)[5]	*
	GERGFFYTPKA	B 20-30	1288,6277	1288,6321	-0,0044	-3	Oxidation (F)[4] or Oxidation (Y)[5]	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3562,7529	3562,7504	0,0025	1	Glycation (F)[1] or Glycation (K)[29]	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3578,7585	3578,7478	0,0107	3	Glycation (F)[1] or Glycation (K)[29], Oxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5894,6517	5894,6774	-0,0257	-4	Glycation (F)[1] or Glycation (K)[29]	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5910,6009	5910,6723	-0,0714	-12	Glycation (F)[1] or Glycation (K)[29], Oxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5926,6116	5926,6672	-0,0556	-9	Glycation (F)[1] or Glycation (K)[29], Dioxidation	
24h								
	RGFFYTPKA	B 22-30	1086,5533	1086,5731	-0,0198	-18		*
	RGFFYTPKA	B 22-30	1102,5420	1102,5680	-0,0260	-24	Oxidation (F)[4] or Oxidation (Y)[5]	*
	ERGFFYTPKA	B 21-30	1215,5890	1215,6157	-0,0267	-22		*

ERGFFYTPKA	B 21-30	1231,5986	1231,6106	-0,0120	-10	Oxidation (F)[5] or Oxidation (Y)[6]	*
RGFFYTPKA	B 22-30	1250,6138	1250,6421	-0,0283	-23	Glycation (K)[8]	*
GERGFFYTPKA	B 20-30	1272,6420	1272,6371	0,0049	4		
GERGFFYTPKA	B 20-30	1288,6524	1288,6321	0,0203	16	Oxidation (F)[5 or 6]	
ERGFFYTPKA	B 21-30	1379,6578	1379,6847	-0,0269	-19	Glycation (K)[8]	*
GERGFFYTPKA	B 20-30	1436,6795	1436,7062	-0,0267	-19	Glycation (K)[8]	
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3562,6865	3562,7504	-0,0639	-18	Glycation (F)[1] or Glycation (K)[29]	
FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3578,7918	3578,7478	0,0440	12	Glycation (F)[1] or Glycation (K)[29], Oxidation	
GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	5896,6802	5896,6930	-0,0128	-2	Glycation (F)[1] or Glycation (K)[29], only two disulfide bridges	

48h

GERGFFYTP	B 20-28	1073,5292	1073,5056	0,0236	22		
GERGFFYTPK	B 20-29	1201,6165	1201,6006	0,0159	13		
ERGFFYTPKA	B 21-30	1215,6340	1215,6157	0,0183	15		*
ERGFFYTPKA	B 21-30	1231,6012	1231,6106	-0,0094	-8	Oxidation (P)[8]	*
CGERGFFYTP	B 19-28	1240,5107	1240,4939	0,0168	14	Cysteic acid (C)[1], Oxidation (Y)[8] or Oxidation (P)[10]	
FVNQHLCGSHL	B 1-11	1254,6332	1254,6053	0,0279	22		
GERGFFYTPKA	B 20-30	1272,6567	1272,6377	0,0190	15		
GERGFFYTPKA	B 20-30	1286,6444	1286,6170	0,0274	21	Pro->pyro-glutamic acid (P)[9]	
GERGFFYTPKA	B 20-30	1288,6483	1288,6321	0,0162	13	Oxidation (F)[6]	
SHLVEALYLVC	B 9-19	1294,6186	1294,6348	-0,0162	-13	Cysteic acid (C)[11]	

GERGFFYTPKA	B 20-30	1300,5855	1300,5962	-0,0107	-8	Oxidation (Y)[7], Pro->pyro-glutamic acid (P)[9]	
GERGFFYTPKA	B 20-30	1302,6342	1302,6113	0,0229	18	Oxidation (F)[6], Pro->pyro-glutamic acid (P)[9]	
CGERGFFYTPK	B 19-30	1304,6233	1304,6098	0,0135	10		
GERGFFYTPKA	B 20-30	1314,5503	1314,5755	-0,0252	-19	Oxidation (G)[1], Pro->Pyrrolidone (P)[9]	
GERGFFYTPKA	B 20-30	1316,6113	1316,5906	0,0207	16	Oxidation (F)[6], Pro->pyro-glutamic acid (P)[9] and Oxidation (G)[4]	
CGERGFFYTPK	B 19-29	1320,6260	1320,6041	0,0219	17	Oxidation (Y)[8] or Oxidation (P)[10]	
GERGFFYTPKA	B 20-30	1332,6031	1332,5855	0,0176	13	Oxidation (F)[5,6], Pro->pyro-glutamic acid (P)[9] and Oxidation (G)[4]	
CGERGFFYTPK	B 19-29	1336,6272	1336,5990	0,0282	21	Oxidation (Y)[8] and Oxidation (P)[10]	
GERGFFYTPK	B 20-29	1365,6761	1365,6691	0,0070	5	Glycation (K)[10]	
CGERGFFYTPKA	B 19-30	1375,6678	1375,6469	0,0209	15		
YLVCGERGFFY	B 16-26	1385,6453	1385,6194	0,0259	19	Oxidation (Y)[1,11]	*
CGSHLVEALYLVC	B 7-19	1406,6578	1406,6807	-0,0229	-16		
GERGFFYTPKA	B 20-30	1436,7097	1436,7062	0,0035	2	Glycation (K)[10]	
GERGFFYTPKA	B 20-30	1450,7169	1450,6855	0,0314	22	Glycation (K)[10], Pro->pyro-glutamic acid (P)[9]	
GERGFFYTPKA	B 20-30	1452,7024	1452,7011	0,0013	1	Glycation (K)[10], Oxidation (F)[5]	
LYLVCGERGFFY	B 15-26	1466,7141	1466,7142	-0,0001	0		*
VCGERGFFYTPKA	B 18-30	1474,7166	1474,7153	0,0013	1		
FVNQHLCGSHLVE	B 1-13	1482,6821	1482,7163	-0,0342	-23		*

FVNQHLCGSHLVE	B 1-13	1558,6913	1558,6596	0,0317	20	Oxidation (H)[5], Cysteic acid (C)[7] and Oxidation (G)[8]	*
LVCGERGFFYTPKA	B 17-30	1587,7998	1587,7994	0,0004	0		*
VCGERGFFYTPKA	B 18-30	1638,7721	1638,7838	-0,0117	-7	Glycation (K)[12]	
VCGERGFFYTPKA	B 18-30	1652,7327	1652,7631	-0,0304	-18	Glycation (K)[12], Pro->pyro-glutamic acid (P)[11]	
YLVCGERGFFYTPKA	B 16-30	1750,8680	1750,8627	0,0053	3		
YLVCGERGFFYTPKA	B 16-30	1764,8707	1764,8420	0,0287	16	Pro->pyro-glutamic acid (P)[13]	
YLVCGERGFFYTPKA	B 16-30	1914,9156	1914,9312	-0,0156	-8	Glycation (K)[14]	
HLVEALYLVCGERGFFYTPKA	B 11-30	2440,2503	2440,2475	0,0028	1		

Sample	Sequence	Position	Observed Mass	Calculated Mass	Match Error Da	Match Error PPM	Modification	Glu-C digests
Red2g 4h								
	ERGFFYTPKA	B 21-30	1215,5918	1215,6157	-0,0239	-20		*
	GERGFFYTPKA	B 20-30	1272,6096	1272,6377	-0,0281	-22		
	GERGFFYTPKA	B 20-30	1288,5943	1288,6321	-0,0378	-29	Oxidation (F)[6]	
	ERGFFYTPKA	B 21-30	1379,6650	1379,6847	-0,0197	-14	Glycation (K)[9]	*
	LCGSHLVEALYLVC	B 6-19	1519,7811	1519,7653	0,0158	10		
	CGSHLVEALYLVCGE	B 7-21	1592,7483	1592,7453	0,0030	2		*
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3562,8004	3562,7504	0,0500	14	Glycation (F)[1] or Glycation (K)[29]	

	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3578,8162	3578,7478	0,0684	19	Glycation (F)[1] or Glycation (K)[29], Oxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6058,9035	6058,7458	0,1577	26	Diglycation - in two of these positions: (F)[1] or (K)[29] or (G)[1]	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6074,7581	6074,7407	0,0174	3	Diglycation - in two of these positions: (F)[1] or (K)[29] or (G)[1], Oxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6090,7849	6090,7356	0,0493	8	Diglycation - in two of these positions: (F)[1] or (K)[29] or (G)[1], Dioxidation	
24h								
	RGFFYTPKA	B 22-30	1086,5543	1086,5731	-0,0188	-17		*
	RGFFYTPKA	B 22-30	1102,5527	1102,5680	-0,0153	-14	Oxidation (F)[4] or Oxidation (Y)[5]	*
	ERGFFYTPKA	B 21-30	1215,6016	1215,6157	-0,0141	-12		*
	ERGFFYTPKA	B 21-30	1231,6127	1231,6106	0,0021	2	Oxidation (F)[5] or Oxidation (Y)[6]	*
	RGFFYTPKA	B 22-30	1250,6245	1250,6421	-0,0176	-14	Glycation (K)[8]	*
	RGFFYTPKA	B 22-30	1266,6422	1266,6370	0,0052	4	Glycation (K)[8], Oxidation (F)[4] or Oxidation (Y)[5]	*
	GERGFFYTPKA	B 20-30	1272,6367	1272,6377	-0,0010	-1		
	GERGFFYTPKA	B 20-30	1288,6607	1288,6321	0,0286	22	Oxidation (F)[6]	
	ERGFFYTPKA	B 21-30	1379,6698	1379,6847	-0,0149	-11	Glycation (K)[9]	*
	ERGFFYTPKA	B 21-30	1395,6743	1395,6796	-0,0053	-4	Glycation (K)[9], Oxidation (Y)[6]	*
	GERGFFYTPKA	B 20-30	1436,7115	1436,7062	0,0053	4	Glycation (K)[10]	
	GIVEQCCASVCSLYQLENYCN	A 1-21	2501,1150	2501,0345	0,0805	32	Glycation (G)[1]	

	GIVEQCCASVCSLYQLENYCN	A 1-21	2517,0615	2517,0294	0,0321	13	Glycation (G)[1], Oxidation	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3578,8113	3578,7478	0,0635	18	Glycation (F)[1] or Glycation (K)[29], Oxidation	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3594,7366	3594,7427	-0,0061	-2	Glycation (F)[1] or Glycation (K)[29], Dioxidation	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3726,8159	3726,8188	-0,0029	-1	Glycation (F)[1] and Glycation (K)[29]	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3758,8623	3758,8086	0,0537	14	Glycation (F)[1] and Glycation (K)[29], Dioxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6058,8133	6058,7458	0,0675	11	Diglycation - in two of these positions: (F)[1] or (K)[29] or (G)[1]	
48h								
	LVCGERG	B 17-23	733,3766	733,3667	0,0099	13		*
	VCGERGF	B 18-24	767,3800	767,3510	0,0290	38		
	LVCGERGF	B 17-24	880,4506	880,4351	0,0155	18		*
	YLVCGERG	B 16-23	896,4412	896,4300	0,0112	12		
	LCGSHLVEA	B 6-14	928,4477	928,4562	-0,0085	-9		*
	YLVCGERGF	B 16-24	1043,5100	1043,4984	0,0116	11		
	HLCGSHLVEAL	B 5-15	1178,5709	1178,5992	-0,0283	-24		*
	GIVEQCCASVCS	A 1-12	1374,5337	1374,5228	0,0109	8	Glycation (G)[1] and Oxidation (G)[1]	*
	ASVCSLYQLENY	A 8-19	1389,5994	1389,6360	-0,0366	-26		
	ASVCSLYQLENY	A 8-19	1405,5995	1405,6309	-0,0314	-22	Oxidation (C)[4]	
	ASVCSLYQLENY	A 8-19	1421,6031	1421,6258	-0,0227	-16	Oxidation (C)[4], Oxidation (Y)[7]	

SHLVEALYLVCGE	B 9-21	1432,7078	1432,7146	-0,0068	-5		*
SHLVEALYLVCGE	B 9-21	1448,7173	1448,7095	0,0078	5	2-oxohistidine (H)[2]	*
SVCSLYQLENYCN	A 9-21	1535,6255	1535,6509	-0,0254	-17		
GIVEQCCASVCSLY	A 1-14	1538,6130	1538,6177	-0,0047	-3	Oxidation (V)[3], Cysteic acid (C)[6]	
LCGSHLVEALYLVCG	B 6-20	1576,7728	1576,7868	-0,0140	-9		*
LCGSHLVEALYLVCG	B 6-20	1590,7764	1590,7660	0,0104	7	Oxidation (H)[5]	*
ASVCSLYQLENYCN	A 8-21	1606,6974	1606,6882	0,0092	6		
LCGSHLVEALYLVC	B 6-19	1631,7287	1631,7297	-0,0010	-1	Cysteic acid (C)[2,14], Oxidation (L)[10]	*
LYLVCGERGFFYTP	B 15-28	1664,7931	1664,8147	-0,0216	-13		*
FVNQHLCGSHLVEAL	B 1-15	1680,7808	1680,8168	-0,0360	-21	Oxidation (H)[10]	
VNQHLCGSHLVEALY	B 2-16	1682,8098	1682,8324	-0,0226	-13		*
CCASVCSLYQLENYC	A 6-20	1698,6925	1698,6636	0,0289	17		*
GSHLVEALYLVCGERG	B 8-20	1702,8490	1702,8587	-0,0097	-6		
HLCGSHLVEALYLVCG	B 5-20	1713,8757	1713,8457	0,0300	18		*
HLCGSHLVEALYLVCG	B 5-20	1729,8729	1729,8406	0,0324	19	Oxidation (G)[4]	*
ALYLVCGERGFFYTP	B 14-28	1735,8284	1735,8518	-0,0234	-13		*
HLCGSHLVEALYLVCG	B 5-20	1743,8627	1743,8199	0,0428	25	Oxidation (L)[7], Oxidation (G)[4]	*
GSHLVEALYLVCGERG	B 8-20	1750,8584	1750,8434	0,0150	9	Cysteic acid (C)[12]	
CCASVCSLYQLENYC	A 6-20	1760,6760	1760,6276	0,0484	27	Cysteic acid (C)[6], Oxidation (L)[8]	*
HLCGSHLVEALYLVCG	B 5-20	1761,8778	1761,8304	0,0474	27	Cysteic acid (C)[3]	*
CCASVCSLYQLENYC	A 6-20	1776,6755	1776,6225	0,0530	30	Cysteic acid (C)[6], Oxidation (L)[8], Oxidation (Y)[9]	*
GIVEQCCASVCSLYQL	A 1-16	1779,7615	1779,7603	0,0012	1	Oxidation (V)[3], Cysteic acid (C)[6]	
CCASVCSLYQLENYC	A 6-20	1792,6765	1792,6174	0,0591	33	Oxidation (V)[5], Cysteic acid (C)[6], Oxidation (L)[8], Oxidation (Y)[9]	*

NQHLCGSHLVEALYLV	B 3-18	1795,9436	1795,9165	0,0271	15		
QHLCGSHLVEALYLVCG	B 4-20	1889,8783	1889,8890	-0,0107	-6	Cysteic acid (C)[4]	*
YLVCGERGFFYTPK	B 16-29	1923,8303	1923,8686	-0,0383	-20	Cysteic acid (C)[4], Oxidation (F)[9], Oxidation (Y)[11], Glycation (K)[14]	
SHLVEALYLVCGERGFF	B 9-25	1939,9354	1939,9740	-0,0386	-20		*
QCCASVCSLYQLENYCN	A 5-21	1940,8142	1940,7651	0,0491	25		*
CGSHLVEALYLVCGERGF	B 7-24	1952,8974	1952,9363	-0,0389	-20		
SHLVEALYLVCGERGFF	B 9-25	1955,9433	1955,9689	-0,0256	-13	Oxidation (F)[17]	*
QHLCGSHLVEALYLVCGE	B 4-21	1986,8905	1986,9417	-0,0512	-26	2-oxohistidine (H)[7]	*
EQCCASVCSLYQLENYC	A 4-20	1987,7980	1987,7545	0,0435	22	Oxidation (C)[4], Oxidation (V)[7]	
CGSHLVEALYLVCGERGF	B 7-24	2000,8951	2000,9210	-0,0259	-13	Cysteic acid (C)[1]	
CGSHLVEALYLVCGERGF	B 7-24	2016,8954	2016,9159	-0,0205	-10	Cysteic acid (C)[1], Oxidation (L)[5]	
GIVEQCCASVCSLYQLENYCN	A 1-21	2336,9404	2336,9660	-0,0256	-11		
GIVEQCCASVCSLYQLENYCN	A 1-21	2517,0261	2517,0294	-0,0033	-1	Glycation (G)[1], Oxidation (V)[3]	

Sample	Sequence	Position	Observed Mass	Calculated Mass	Match Error Da	Match Error PPM	Modification	Glu-C digests
Red3g 4h								
	RGFFYTPKA	B 22-30	1250,6361	1250,6421	-0,0060	-5	Glycation (K)[8]	*
	GERGFFYTPKA	B 20-30	1272,6232	1272,6377	-0,0145	-11		
	GERGFFYTPKA	B 20-30	1288,6460	1288,6321	0,0139	11	Oxidation (Y)[7]	

	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3578,7195	3578,7478	-0,0283	-8	Glycation (F)[1] or Glycation (K)[29], Oxidation	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3726,8237	3726,8188	0,0049	1	Glycation (F)[1] and Glycation (K)[29]	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-30	3742,7338	3742,8137	-0,0799	-21	Glycation (F)[1] and Glycation (K)[29], Oxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6223,1016	6222,9042	0,1975	32	Triglycation: (F)[1], (K)[29], (G)[1]	
24h								
	ERGFFYTPKA	B 21-30	1215,6272	1215,6157	0,0115	9		*
	RGFFYTPKA	B 22-30	1250,6537	1250,6421	0,0116	9	Glycation (K)[8]	*
	RGFFYTPKA	B 22-30	1266,6593	1266,6370	0,0223	18	Glycation (K)[8], Oxidation (F)[4] or Oxidation (Y)[5]	*
	GERGFFYTPKA	B 20-30	1272,6077	1272,6377	-0,0300	-24		
	GERGFFYTPKA	B 20-30	1288,6674	1288,6321	0,0353	27	Oxidation (F)[6]	
	ERGFFYTPKA	B 21-30	1379,6795	1379,6847	-0,0052	-4	Glycation (K)[9]	*
	ERGFFYTPKA	B 21-30	1395,6822	1395,6796	0,0026	2	Glycation (K)[9], Oxidation (Y)[6]	*
	GERGFFYTPKA	B 20-30	1436,7273	1436,7062	0,0212	15	Glycation (K)[10]	
	GIVEQCCASVCSLYQLENYCN	A 1-21	2516,9828	2517,0294	-0,0466	-19	Glycation (G)[1], Oxidation	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3576,7400	3576,7322	0,0078	2	Glycation (F)[1] or Glycation (K)[29], Oxidation	
	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	B 1-31	3726,8536	3726,8188	0,0348	9	Glycation (F)[1] and Glycation (K)[29]	

	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6238,9450	6238,8991	0,0459	7	Triglycation - (F)[1], (K)[29], (G)[1]; Oxidation	
	GIVEQCCASVCSLYQLENYCN / FVNQHLCGSHLVEALYLVCGERGFFYTPKA	A 1-21 / B 1-30	6255,0165	6254,8940	0,1225	20	Triglycation - (F)[1], (K)[29], (G)[1]; Dioxidation	
48h								
	LVCGERG	B 17-23	733,3622	733,3667	-0,0045	-6		*
	LVCGERGF	B 17-24	880,4404	880,4351	0,0053	6		*
	YLVCGERG	B 16-23	896,4482	896,4300	0,0182	20		
	LCGSHLVEA	B 6-14	928,4612	928,4562	0,0050	5		*
	YLVCGERGF	B 16-24	1043,5149	1043,4984	0,0165	16		
	VCSLYQLEN	A 10-18	1132,5085	1132,4832	0,0253	22	Oxidation (V)[1], Cysteic acid (C)[2]	
	HLCGSHLVEAL	B 5-15	1178,6070	1178,5992	0,0078	7		*
	GIVEQCCASVCS	A 1-12	1374,5277	1374,5228	0,0049	4	Glycation (G)[1] and Oxidation (G)[1]	*
	ERGFFYTPKA	B 21-30	1379,6731	1379,6847	-0,0116	-8	Glycation (K)[9]	*
	ASVCSLYQLENY	A 8-19	1389,6014	1389,6360	-0,0346	-25		
	ASVCSLYQLENY	A 8-19	1405,6027	1405,6309	-0,0282	-20	Oxidation (C)[4]	
	ASVCSLYQLENY	A 8-19	1421,6045	1421,6258	-0,0213	-15	Oxidation (C)[4], Oxidation (Y)[7]	
	GERGFFYTPKA	B 20-30	1436,7128	1436,7062	0,0067	5	Glycation (K)[10]	
	CASVCSLYQLENY	A 7-19	1492,6257	1492,6451	-0,0194	-13		
	CASVCSLYQLENY	A 7-19	1506,6273	1506,6244	0,0029	2	Oxidation (L)[10)	
	CASVCSLYQLENY	A 7-19	1520,6251	1520,6037	0,0214	14	Oxidation (L)[10), Oxidation (Y)[13]	
	SVCSLYQLENYCN	A 9-21	1535,6194	1535,6509	-0,0315	-21		

CASVCSLYQLENY	A 7-19	1536,6223	1536,5986	0,0237	15	Oxidation (Y)[8], Oxidation (L)[10], Oxidation (Y)[13]	
GIVEQCCASVCSLY	A 1-14	1538,6043	1538,6177	-0,0134	-9	Oxidation (V)[3], Cysteic acid (C)[6]	
SVCSLYQLENYCN	A 9-21	1551,6329	1551,6458	-0,0129	-8	Oxidation (V)[2]	
LCGSHLVEALYLVC	B 6-19	1631,7458	1631,7297	0,0161	10	Cysteic acid (C)[2,14], Oxidation (L)[10]	
GIVEQCCASVCSLY	A 1-14	1638,7166	1638,7065	0,0101	6	Glycation (G)[1]	
VCGERGFFYTPKA	B 18-30	1638,7924	1638,7838	0,0086	5	Glycation (K)[12]	
LYLVCGERGFFYTP	B 15-28	1664,8087	1664,8147	-0,0060	-4		*
VNQHLCGSHLVEALY	B 2-16	1682,7956	1682,8324	-0,0368	-22		*
CCASVCSLYQLENYC	A 6-20	1698,6853	1698,6636	0,0217	13		*
GSHLVEALYLVCGERG	B 8-20	1702,8449	1702,8587	-0,0138	-8		
HLCGSHLVEALYLVCG	B 5-20	1713,8587	1713,8457	0,0130	8		*
HLCGSHLVEALYLVCG	B 5-20	1729,8635	1729,8406	0,0230	13	Oxidation (G)[4]	*
HLCGSHLVEALYLVCG	B 5-20	1743,8598	1743,8199	0,0399	23	Oxidation (L)[7], Oxidation (G)[4]	*
GSHLVEALYLVCGERG	B 8-20	1750,8411	1750,8434	-0,0023	-1	Cysteic acid (C)[12]	
LVCGERGFFYTPKA	B 17-30	1751,8357	1751,8679	-0,0322	-18	Glycation (K)[13]	*
CCASVCSLYQLENYC	A 6-20	1760,6699	1760,6276	0,0423	24	Cysteic acid (C)[6], Oxidation (L)[8]	*
CCASVCSLYQLENYC	A 6-20	1776,6646	1776,6225	0,0421	24	Cysteic acid (C)[6], Oxidation (L)[8], Oxidation (Y)[9]	*
CCASVCSLYQLENYC	A 6-20	1792,6626	1792,6174	0,0452	25	Oxidation (V)[5], Cysteic acid (C)[6], Oxidation (L)[8], Oxidation (Y)[9]	*
NQHLCGSHLVEALYLV	B 3-18	1795,9426	1795,9165	0,0261	15		
CCASVCSLYQLENYC	A 6-20	1810,6717	1810,6280	0,0437	24	Cysteic acid (C)[6,15], Oxidation (Y)[14]	*
FVNQHLCGSHLVEAL	B 1-15	1830,8909	1830,9060	-0,0151	-8	Glycation (F)[1]	

FVNQHLCGSHLVEAL	B 1-15	1844,8882	1844,8852	0,0030	2	Glycation (F)[1], Oxidation (F)[1]	
YLVCGERGFFYTPKA	B 16-30	1914,9498	1914,9312	0,0186	10	Glycation (K)[14]	
YLVCGERGFFYTPK	B 16-29	1923,8263	1923,8686	-0,0423	-22	Cysteic acid (C)[4], Oxidation (F)[9], Oxidation (Y)[11], Glycation (K)[14]	
SHLVEALYLVCGERGFF	B 9-25	1939,9280	1939,9740	-0,0460	-24		*
CGSHLVEALYLVCGERGF	B 7-24	1952,9031	1952,9363	-0,0332	-17		
SHLVEALYLVCGERGFF	B 9-25	1955,9196	1955,9689	-0,0493	-25	Oxidation (F)[17]	*
QHLCGSHLVEALYLVCGE	B 4-21	1984,8988	1984,9261	-0,0273	-14	Oxidation (G)[5]	*
QHLCGSHLVEALYLVCGE	B 4-21	1986,9002	1986,9417	-0,0415	-21	2-oxohistidine (H)[7]	*
EQCCASVCSLYQLENYC	A 4-20	1987,7889	1987,7545	0,0344	17	Oxidation (C)[4], Oxidation (V)[7]	
CGSHLVEALYLVCGERGF	B 7-24	2000,9016	2000,9210	-0,0194	-10	Cysteic acid (C)[1]	
CGSHLVEALYLVCGERGF	B 7-24	2016,9019	2016,9159	-0,0140	-7	Cysteic acid (C)[1], Oxidation (L)[5]	
ALYLVCGERGFFYTPKA	B 14-30	2130,9836	2131,0422	-0,0586	-27	Oxidation (C)[6], Oxidation (F)[11], Glycation (K)[16]	*
ALYLVCGERGFFYTPKA	B 14-30	2146,9879	2147,0371	-0,0492	-23	Oxidation (C)[6], Oxidation (F)[11], Oxidation (P)[15], Glycation (K)[16]	*
ALYLVCGERGFFYTPKA	B 14-30	2160,9438	2161,0164	-0,0726	-34	Oxidation (V)[5], Oxidation (C)[6], Oxidation (F)[11], Pro->pyro-glutamic acid (P)[15], Glycation (K)[16]	*
ALYLVCGERGFFYTPKA	B 14-30	2162,9443	2163,0320	-0,0877	-41	Oxidation (V)[5], Oxidation (C)[6], Oxidation (F)[11], Oxidation (P)[15], Glycation (K)[16]	*
FVNQHLCGSHLVEALYLV	B 1-18	2206,1290	2206,1218	0,0072	3	Glycation (F)[1]	
HLCGSHLVEALYLVCGERGF	B 5-24	2250,9726	2251,0639	-0,0913	-41	Cysteic acid (C)[15]	*
GIVEQCCASVCSLYQLENYCN	A 1-21	2336,9385	2336,9660	-0,0275	-12		

GIVEQCCASVCSLYQLENYCN	A 1-21	2354,9398	2354,9766	-0,0368	-16	Oxidation (C)[11]
GIVEQCCASVCSLYQLENYCN	A 1-21	2516,9691	2517,0294	-0,0603	-24	Glycation (G)[1], Oxidation (V)[3]
HLVEALYLVCGERGFFYTPKA	B 10-30	2554,3196	2554,2904	0,0292	11	His->Asn (H)[1], Glycation (K)[20]
HLVEALYLVCGERGFFYTPKA	B 10-30	2568,3087	2568,2696	0,0391	15	His->Asn (H)[1], Oxidation (L)[2], Glycation (K)[20]
FVNQHLCGSHLVEALYLVCGER	B 1-22	2651,2893	2651,2961	-0,0068	-3	Glycation (F)[1]
FVNQHLCGSHLVEALYLVCGER	B 1-22	2665,2803	2665,2754	0,0048	2	Glycation (F)[1], Oxidation (F)[1]

Paper 4 *supplemental tables*

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B Control	2020,1913	2020,1748	-0,0165	-8	2	21	PEPAKSAPAPKKGSKKAVTK	
	1265,6412	1265,6362	-0,005	-4	35	44	KESYSVYVYK	
	1605,8887	1605,8584	-0,0303	-19	35	47	KESYSVYVYKVLK	
	1674,9214	1674,8951	-0,0263	-16	39	52	SVYVYKVLKQVHPD	
	1587,8893	1587,8713	-0,018	-11	40	52	VYVYKVLKQVHPD	
	1899,0698	1899,0508	-0,019	-10	42	58	VYKVLKQVHPDTGISSK	
	2341,2366	2341,2053	-0,0313	-13	44	65	KVLKQVHPDTGISSKAMGIMNS	
	1744,8357	1744,8081	-0,0276	-16	49	65	VHPDTGISSKAMGIMNS	
	1459,6919	1459,6716	-0,0203	-14	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
	1208,7725	1208,7900	0,0175	14	99	109	VRLLLPGELAK	
	1476,8420	1476,8250	-0,017	-12	101	114	LLLPGELAKHAVSE	
	1363,7579	1363,7440	-0,0139	-10	102	114	LLPGELAKHAVSE	
	1270,7002	1270,7014	0,0012	1	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B Ox_1 - 30min	1892,0963	1892,0793	-0,017	-9	2	20	PEPAKSAPAPKKGSKKAVT	
	875,4145	875,4368	0,0223	25	35	41	KESYSVY	
	974,4828	974,4799	-0,0029	-3	35	42	KESYSVYV	
	1137,5463	1137,5492	0,0029	3	35	43	KESYSVYVY	
	1220,6561	1220,6400	-0,0161	-13	37	46	SYSVYVYKVL	
	1348,7511	1348,7422	-0,0089	-7	37	47	SYSVYVYKVLK	
	970,5607	970,5667	0,006	6	39	46	SVYVYKVL	
	1325,7576	1325,7338	-0,0238	-18	42	52	VYKVLKQVHPD	
	2229,1365	2229,0916	-0,0449	-20	45	65	VLKQVHPDTGISSKAMGIMNS	Oxidation (N)[20]
	2229,1365	2229,0916	-0,0449	-20	45	65	VLKQVHPDTGISSKAMGIMNS	Oxidation (M)[19]

	2229,1365	2229,0916	-0,0449	-20	45	65	VLKQVHPDTGISSKAMGIMNS	Oxidation (K)[14]
	2360,2100	2360,1833	-0,0267	-11	45	66	VLKQVHPDTGISSKAMGIMNSF	
	2376,2051	2376,1682	-0,0369	-16	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
	1443,6970	1443,6891	-0,0079	-5	53	66	TGISSKAMGIMNSF	
	1459,6919	1459,6764	-0,0155	-11	53	66	TGISSKAMGIMNSF	Oxidation (F)[14]
	998,4797	998,4860	0,0063	6	58	66	KAMGIMNSF	
	1326,6180	1326,6180	0	0	58	69	KAMGIMNSFVND	
	870,3848	870,3760	-0,0088	-10	59	66	AMGIMNSF	
	1039,5208	1039,5317	0,0109	10	66	73	FVNDIFER	
	1262,6376	1262,6462	0,0086	7	67	77	VNDIFERIAGE	
	934,4992	934,5233	0,0241	26	70	77	IFERIAGE	
	1248,6694	1248,6731	0,0037	3	70	80	IFERIAGEASR	
	1187,6167	1187,6244	0,0077	6	74	84	IAGEASRLAHY	
	1215,6705	1215,6831	0,0126	10	78	87	ASRLAHYNKR	
	1279,8096	1279,8159	0,0063	5	98	109	AVRLLLPGELAK	
	1080,6775	1080,7014	0,0239	22	99	108	VRLLLPGELA	
	1208,7725	1208,7816	0,0091	8	99	109	VRLLLPGELAK	
	1515,9369	1515,9338	-0,0031	-2	99	112	VRLLLPGELAKHAV	
	953,6030	953,6179	0,0149	16	101	109	LLLPGELAK	
	1486,7748	1486,7737	-0,0011	-1	113	126	SEGTKAVTKYTSSK	
	1270,7002	1270,7048	0,0046	4	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B	1492,8481	1492,8065	-0,0416	-28	2	16	PEPAKSAPAPKKGSK	
Ox_1 - 1h	1892,0963	1892,0818	-0,0145	-8	2	20	PEPAKSAPAPKKGSKKAVT	
	875,4145	875,4253	0,0108	12	35	41	KESYSVY	
	974,4828	974,4866	0,0038	4	35	42	KESYSVYV	
	1137,5463	1137,5504	0,0041	4	35	43	KESYSVYVY	

1477,7937	1477,7877	-0,006	-4	35	46	KESYSVYVYKVL	
1605,8887	1605,8575	-0,0312	-19	35	47	KESYSVYVYKVLK	
1008,5036	1008,5074	0,0038	4	37	44	SYSVYVYK	
1325,7576	1325,7450	-0,0126	-10	42	52	VYKVLKQVHPD	
2603,3684	2603,3643	-0,0041	-2	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2619,3633	2619,2935	-0,0698	-27	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
2619,3633	2619,2935	-0,0698	-27	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (M)[22]
2619,3633	2619,2935	-0,0698	-27	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (K)[17]
2619,3633	2619,2935	-0,0698	-27	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (P)[10]
2750,4368	2750,4304	-0,0064	-2	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
2766,4316	2766,3879	-0,0437	-16	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[25]
2488,3049	2488,3020	-0,0029	-1	44	66	KVLKQVHPDTGISSKAMGIMNSF	
1508,8431	1508,8441	0,001	1	45	58	VLKQVHPDTGISSK	
2360,2100	2360,1953	-0,0147	-6	45	66	VLKQVHPDTGISSKAMGIMNSF	
2376,2051	2376,1619	-0,0432	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (M)[16]
2376,2051	2376,1614	-0,0437	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1614	-0,0437	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
1799,9142	1799,8857	-0,0285	-16	47	63	KQVHPDTGISSKAMGIM	
2148,0576	2148,0376	-0,02	-9	47	66	KQVHPDTGISSKAMGIMNSF	
2164,0525	2164,0239	-0,0286	-13	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (N)[18]
2164,0525	2164,0239	-0,0286	-13	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (M)[17]
1443,6970	1443,6793	-0,0177	-12	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6769	-0,015	-10	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
1459,6919	1459,6769	-0,015	-10	53	66	TGISSKAMGIMNSF	Oxidation (N)[12]
1459,6919	1459,6952	0,0033	2	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
998,4797	998,4890	0,0093	9	58	66	KAMGIMNSF	
870,3848	870,3862	0,0014	2	59	66	AMGIMNSF	
1039,5208	1039,5350	0,0142	14	66	73	FVNDIFER	
934,4992	934,5251	0,0259	28	70	77	IFERIAGE	
1472,7605	1472,7528	-0,0077	-5	72	84	ERIAGEASRLAHY	
1429,7546	1429,7550	0,0004	0	74	86	IAGEASRLAHYNK	

	1279,8096	1279,8221	0,0125	10	98	109	AVRLLLPGELAK	
	1208,7725	1208,7850	0,0125	10	99	109	VRLLLPGELAK	
	1515,9369	1515,9307	-0,0062	-4	99	112	VRLLLPGELAKHAV	
	1109,7041	1109,7194	0,0153	14	100	109	RLLLPGELAK	
	1476,8420	1476,8274	-0,0146	-10	101	114	LLLPGELAKHAVSE	
	1363,7579	1363,7544	-0,0035	-3	102	114	LLPGELAKHAVSE	
	1486,7748	1486,7531	-0,0217	-15	113	126	SEGTKAVTKYTSSK	
	1269,6686	1269,6472	-0,0214	-17	115	126	GTKAVTKYTSSK	Lys->Allysine (K)[12]
	1270,7002	1270,7045	0,0043	3	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B	875,4145	875,4086	-0,0059	-7	35	41	KESYSVY	
Ox_1 - 3h	974,4828	974,4697	-0,0131	-13	35	42	KESYSVYV	
	1137,5463	1137,5397	-0,0066	-6	35	43	KESYSVYVY	
	1380,7045	1380,7104	0,0059	4	35	45	KESYSVYVYKV	Oxidation (Y)[9]
	1008,5036	1008,4963	-0,0073	-7	37	44	SYSVYVYK	
	1348,7511	1348,7346	-0,0165	-12	37	47	SYSVYVYKVLK	
	1325,7576	1325,7584	0,0008	1	42	52	VYKVLKQVHPD	
	1899,0698	1899,0527	-0,0171	-9	42	58	VYKVLKQVHPDTGISSK	
	1800,0015	1799,9806	-0,0209	-12	43	58	YKVLKQVHPDTGISSK	
	2000,9893	2000,9783	-0,011	-5	47	65	KQVHPDTGISSKAMGIMNS	
	2016,9841	2016,9612	-0,0229	-11	47	65	KQVHPDTGISSKAMGIMNS	Oxidation (M)[14]
	2016,9841	2016,9612	-0,0229	-11	47	65	KQVHPDTGISSKAMGIMNS	Oxidation (N)[18]
	2164,0525	2164,0320	-0,0205	-9	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (F)[20]
	1443,6970	1443,6909	-0,0061	-4	53	66	TGISSKAMGIMNSF	
	1459,6919	1459,6761	-0,0158	-11	53	66	TGISSKAMGIMNSF	Oxidation (F)[14]
	870,3848	870,3712	-0,0136	-16	59	66	AMGIMNSF	
	1039,5208	1039,5341	0,0133	13	66	73	FVNDIFER	

892,4523	892,4537	0,0014	2	67	73	VNDIFER	
934,4992	934,4997	0,0005	1	70	77	IFERIAGE	
1248,6694	1248,6641	-0,0053	-4	70	80	IFERIAGEASR	
1675,8875	1675,8756	-0,0119	-7	81	94	LAHYNKRSTITSRE	
1279,8096	1279,8164	0,0068	5	98	109	AVRLLLPGELAK	
1803,0487	1803,0475	-0,0012	-1	98	114	AVRLLLPGELAKHAVSE	
896,5563	896,5656	0,0093	10	99	106	VRLLLPGE	
1207,7408	1207,7583	0,0175	14	99	109	VRLLLPGELAK	Lys->Allysine (K)[11]
1208,7725	1208,7850	0,0125	10	99	109	VRLLLPGELAK	
1224,7675	1224,7720	0,0045	4	99	109	VRLLLPGELAK	Oxidation (K)[11]
1224,7675	1224,7720	0,0045	4	99	109	VRLLLPGELAK	Oxidation (P)[6]
1224,7675	1224,7720	0,0045	4	99	109	VRLLLPGELAK	Oxidation (L)[9]
1515,9369	1515,9355	-0,0014	-1	99	112	VRLLLPGELAKHAV	
1732,0116	1732,0182	0,0066	4	99	114	VRLLLPGELAKHAVSE	
1109,7041	1109,7111	0,007	6	100	109	RLLLPGELAK	
1632,9431	1632,9447	0,0016	1	100	114	RLLLPGELAKHAVSE	
1476,8420	1476,8331	-0,0089	-6	101	114	LLLPGELAKHAVSE	
953,5050	953,5152	0,0102	11	104	112	PGELAKHAV	Oxidation (V)[9], Oxidation (H)[7]
1270,7002	1270,7081	0,0079	6	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B	875,4145	875,4169	0,0024	3	35	41	KESYSVY	
Ox_1 - 24h	1137,5463	1137,5355	-0,0108	-9	35	43	KESYSVYVY	
	1265,6412	1265,6251	-0,0161	-13	35	44	KESYSVYVYK	
	1605,8887	1605,8601	-0,0286	-18	35	47	KESYSVYVYKVLK	
	1008,5036	1008,4968	-0,0068	-7	37	44	SYSVYVYK	
	2370,2339	2370,2224	-0,0115	-5	37	57	SYSVYVYKVLKQVHPDTGISS	
	1674,9214	1674,8871	-0,0343	-20	39	52	SVYVYKVLKQVHPD	

2120,1387	2120,1042	-0,0345	-16	39	57	SVYVYKVLKQVHPDTGISS	
1011,6237	1011,6135	-0,0102	-10	40	47	VYVYKVLK	
1587,8893	1587,8582	-0,0311	-20	40	52	VYVYKVLKQVHPD	
2033,1066	2033,0873	-0,0193	-9	40	57	VYVYKVLKQVHPDTGISS	
1325,7576	1325,7456	-0,012	-9	42	52	VYKVLKQVHPD	
1770,9749	1770,9534	-0,0215	-12	42	57	VYKVLKQVHPDTGISS	
1899,0698	1899,0509	-0,0189	-10	42	58	VYKVLKQVHPDTGISSK	
2603,3684	2603,3560	-0,0124	-5	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2750,4368	2750,4375	0,0007	0	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
1636,9381	1636,9171	-0,021	-13	44	58	KVLKQVHPDTGISSK	
1293,7162	1293,7009	-0,0153	-12	45	56	VLKQVHPDTGIS	
1380,7482	1380,7522	0,004	3	45	57	VLKQVHPDTGISS	
1508,8431	1508,8364	-0,0067	-4	45	58	VLKQVHPDTGISSK	
1710,9207	1710,8955	-0,0252	-15	45	60	VLKQVHPDTGISSKAM	
2213,1416	2213,1223	-0,0193	-9	45	65	VLKQVHPDTGISSKAMGIMNS	
2360,2100	2360,2007	-0,0093	-4	45	66	VLKQVHPDTGISSKAMGIMNSF	
2376,2051	2376,1765	-0,0286	-12	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2148,0576	2148,0403	-0,0173	-8	47	66	KQVHPDTGISSKAMGIMNSF	
1891,9041	1891,8893	-0,0148	-8	49	66	VHPDTGISSKAMGIMNSF	
1907,8990	1907,8705	-0,0285	-15	49	66	VHPDTGISSKAMGIMNSF	Oxidation (M)[15]
1443,6970	1443,6786	-0,0184	-13	53	66	TGISSKAMGIMNSF	
1085,5118	1085,5072	-0,0046	-4	57	66	SKAMGIMNSF	
1101,5067	1101,5015	-0,0052	-5	57	66	SKAMGIMNSF	Oxidation (M)[4]
1101,5067	1101,5015	-0,0052	-5	57	66	SKAMGIMNSF	Oxidation (K)[2]
1429,6450	1429,6310	-0,014	-10	57	69	SKAMGIMNSFVND	Oxidation (M)[4]
998,4797	998,4824	0,0027	3	58	66	KAMGIMNSF	
1014,4747	1014,4745	-0,0002	0	58	66	KAMGIMNSF	Oxidation (M)[3]
870,3848	870,3719	-0,0129	-15	59	66	AMGIMNSF	
886,3797	886,3751	-0,0046	-5	59	66	AMGIMNSF	Oxidation (M)[5]
886,3797	886,3751	-0,0046	-5	59	66	AMGIMNSF	Oxidation (N)[6]
1039,5208	1039,5332	0,0124	12	66	73	FVNDIFER	

	892,4523	892,4565	0,0042	5	67	73	VNDIFER
	934,4992	934,4969	-0,0023	-2	70	77	IFERIAGE
	1092,5684	1092,5632	-0,0052	-5	70	79	IFERIAGEAS
	1248,6694	1248,6676	-0,0018	-1	70	80	IFERIAGEASR
	1675,8875	1675,8779	-0,0096	-6	81	94	LAHYNKRSTITSRE
	1279,8096	1279,8126	0,003	2	98	109	AVRLLLPGELAK
	1803,0487	1803,0466	-0,0021	-1	98	114	AVRLLLPGELAKHAVSE
	1208,7725	1208,7833	0,0108	9	99	109	VRLLLPGELAK
	1345,8314	1345,8309	-0,0005	0	99	110	VRLLLPGELAKH
	1515,9369	1515,9412	0,0043	3	99	112	VRLLLPGELAKHAV
	1732,0116	1732,0101	-0,0015	-1	99	114	VRLLLPGELAKHAVSE
	1109,7041	1109,7081	0,004	4	100	109	RLLLPGELAK
	1632,9431	1632,9459	0,0028	2	100	114	RLLLPGELAKHAVSE
	953,6030	953,5980	-0,005	-5	101	109	LLLPGELAK
	1476,8420	1476,8257	-0,0163	-11	101	114	LLLPGELAKHAVSE
	1270,7002	1270,7100	0,0098	8	115	126	GTKAVTKYTSSK

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B Ox_2 - 30min	875,4145	875,4371	0,0226	26	35	41	KESYSVY	
	974,4828	974,4790	-0,0038	-4	35	42	KESYSVYV	
	1137,5463	1137,5493	0,003	3	35	43	KESYSVYVY	
	1265,6412	1265,6262	-0,015	-12	35	44	KESYSVYVYK	
	1605,8887	1605,8662	-0,0225	-14	35	47	KESYSVYVYKVLK	
	1008,5036	1008,5092	0,0056	6	37	44	SYSVYVYK	
	1220,6561	1220,6416	-0,0145	-12	37	46	SYSVYVYKVL	
	1348,7511	1348,7417	-0,0094	-7	37	47	SYSVYVYKVLK	
	970,5607	970,5646	0,0039	4	39	46	SVYVYKVL	
	1098,6558	1098,6503	-0,0055	-5	39	47	SVYVYKVLK	

1011,6237	1011,6180	-0,0057	-6	40	47	VYVYKVLK	
1325,7576	1325,7428	-0,0148	-11	42	52	VYKVLKQVHPD	
1899,0698	1899,0404	-0,0294	-15	42	58	VYKVLKQVHPDTGISSK	
2667,3633	2667,3262	-0,0371	-14	43	66	YKVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[24]
2488,3049	2488,2805	-0,0244	-10	44	66	KVLKQVHPDTGISSKAMGIMNSF	
2504,3000	2504,2429	-0,0571	-23	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[23]
2504,3000	2504,2429	-0,0571	-23	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[15]
1508,8431	1508,8358	-0,0073	-5	45	58	VLKQVHPDTGISSK	
2213,1416	2213,1113	-0,0303	-14	45	65	VLKQVHPDTGISSKAMGIMNS	
2229,1365	2229,0999	-0,0366	-16	45	65	VLKQVHPDTGISSKAMGIMNS	Oxidation (K)[14]
2229,1365	2229,0999	-0,0366	-16	45	65	VLKQVHPDTGISSKAMGIMNS	Oxidation (N)[20]
2360,2100	2360,1895	-0,0205	-9	45	66	VLKQVHPDTGISSKAMGIMNSF	
2376,2051	2376,1711	-0,034	-14	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1711	-0,034	-14	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2148,0576	2148,0376	-0,02	-9	47	66	KQVHPDTGISSKAMGIMNSF	
2180,0474	2179,9939	-0,0535	-25	47	66	KQVHPDTGISSKAMGIMNSF	Dioxidation (M)[14]
2180,0474	2179,9939	-0,0535	-25	47	66	KQVHPDTGISSKAMGIMNSF	Dioxidation (F)[20]
1443,6970	1443,6855	-0,0115	-8	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6830	-0,0089	-6	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
1459,6919	1459,7112	0,0193	13	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
1459,6919	1459,6830	-0,0089	-6	53	66	TGISSKAMGIMNSF	Oxidation (F)[14]
886,3797	886,3846	0,0049	6	59	66	AMGIMNSF	Oxidation (M)[5]
886,3797	886,3863	0,0066	7	59	66	AMGIMNSF	Oxidation (M)[2]
886,3797	886,3846	0,0049	6	59	66	AMGIMNSF	Oxidation (N)[6]
902,3746	902,3867	0,0121	13	59	66	AMGIMNSF	Oxidation (M)[2,5]
1039,5208	1039,5378	0,017	16	66	73	FVNDIFER	
892,4523	892,4749	0,0226	25	67	73	VNDIFER	
1262,6376	1262,6461	0,0085	7	67	77	VNDIFERIAGE	
934,4992	934,5201	0,0209	22	70	77	IFERIAGE	
1248,6694	1248,6750	0,0056	4	70	80	IFERIAGEASR	
1187,6167	1187,6068	-0,0099	-8	74	84	IAGEASRLAHY	

	1215,6705	1215,6770	0,0065	5	78	87	ASRLAHYNKR	
	1622,0000	1622,0007	0,0007	0	95	109	IQTAVRLLLPGELAK	
	1279,8096	1279,8246	0,015	12	98	109	AVRLLLPGELAK	
	1803,0487	1803,0454	-0,0033	-2	98	114	AVRLLLPGELAKHAVSE	
	1208,7725	1208,7874	0,0149	12	99	109	VRLLLPGELAK	
	1345,8314	1345,8286	-0,0028	-2	99	110	VRLLLPGELAKH	
	1515,9369	1515,9333	-0,0036	-2	99	112	VRLLLPGELAKHAV	
	1732,0116	1732,0217	0,0101	6	99	114	VRLLLPGELAKHAVSE	
	1109,7041	1109,7238	0,0197	18	100	109	RLLLPGELAK	
	1632,9431	1632,9415	-0,0016	-1	100	114	RLLLPGELAKHAVSE	
	953,6030	953,6199	0,0169	18	101	109	LLLPGELAK	
	1476,8420	1476,8245	-0,0175	-12	101	114	LLLPGELAKHAVSE	
	1363,7579	1363,7462	-0,0117	-9	102	114	LLPGELAKHAVSE	
	1486,7748	1486,7649	-0,0099	-7	113	126	SEGTKAVTKYTSSK	
	1269,6686	1269,6641	-0,0045	-4	115	126	GTKAVTKYTSSK	Lys->Allysine (K)[12]
	1270,7002	1270,7065	0,0063	5	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B	1492,8481	1492,8246	-0,0235	-16	2	16	PEPAKSAPAPKKGSK	
Ox_2 - 1h	2020,1913	2020,1503	-0,041	-20	2	21	PEPAKSAPAPKKGSKKAVTK	
	875,4145	875,4212	0,0067	8	35	41	KESYSVY	
	974,4828	974,4827	-0,0001	0	35	42	KESYSVYV	
	1137,5463	1137,5562	0,0099	9	35	43	KESYSVYVY	
	1265,6412	1265,6309	-0,0103	-8	35	44	KESYSVYVYK	
	1477,7937	1477,7816	-0,0121	-8	35	46	KESYSVYVYKVL	
	1605,8887	1605,8647	-0,024	-15	35	47	KESYSVYVYKVLK	
	1348,7511	1348,7380	-0,0131	-10	37	47	SYSVYVYKVLK	
	1674,9214	1674,8940	-0,0274	-16	39	52	SVYVYKVLKQVHPD	

2248,2336	2248,2019	-0,0317	-14	39	58	SVYVYKVLKQVHPDTGISSK	
1011,6237	1011,6251	0,0014	1	40	47	VYVYKVLK	
1587,8893	1587,8627	-0,0266	-17	40	52	VYVYKVLKQVHPD	
1325,7576	1325,7417	-0,0159	-12	42	52	VYKVLKQVHPD	
1899,0698	1899,0239	-0,0459	-24	42	58	VYKVLKQVHPDTGISSK	
2603,3684	2603,3501	-0,0183	-7	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2619,3633	2619,3267	-0,0366	-14	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (P)[10]
2619,3633	2619,2969	-0,0664	-25	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
2619,3633	2619,2969	-0,0664	-25	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (M)[22]
2619,3633	2619,3091	-0,0542	-21	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (K)[17]
2750,4368	2750,4412	0,0044	2	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
1800,0015	1799,9883	-0,0132	-7	43	58	YKVLKQVHPDTGISSK	
2667,3633	2667,2991	-0,0642	-24	43	66	YKVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[24]
2341,2366	2341,1865	-0,0501	-21	44	65	KVLKQVHPDTGISSKAMGIMNS	
2504,3000	2504,2676	-0,0324	-13	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[8]
2504,3000	2504,2676	-0,0324	-13	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (D)[9]
2504,3000	2504,2446	-0,0554	-22	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[23]
2504,3000	2504,2434	-0,0566	-23	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (M)[17]
2504,3000	2504,2446	-0,0554	-22	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[15]
1508,8431	1508,8422	-0,0009	-1	45	58	VLKQVHPDTGISSK	
2213,1416	2213,1289	-0,0127	-6	45	65	VLKQVHPDTGISSKAMGIMNS	
2360,2100	2360,2002	-0,0098	-4	45	66	VLKQVHPDTGISSKAMGIMNSF	
2376,2051	2376,1826	-0,0225	-9	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1826	-0,0225	-9	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2376,2051	2376,1826	-0,0225	-9	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[7]
2376,2051	2376,1621	-0,043	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (M)[19]
2016,9841	2016,9768	-0,0073	-4	47	65	KQVHPDTGISSKAMGIMNS	Oxidation (M)[17]
2000,9893	2000,9430	-0,0463	-23	47	65	KQVHPDTGISSKAMGIMNS	
2016,9841	2016,9950	0,0109	5	47	65	KQVHPDTGISSKAMGIMNS	Oxidation (M)[14]
2016,9841	2016,9950	0,0109	5	47	65	KQVHPDTGISSKAMGIMNS	Oxidation (K)[12]
2016,9841	2016,9950	0,0109	5	47	65	KQVHPDTGISSKAMGIMNS	Oxidation (N)[18]

1443,6970	1443,6899	-0,0071	-5	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6761	-0,0158	-11	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
1459,6919	1459,6761	-0,0158	-11	53	66	TGISSKAMGIMNSF	Oxidation (N)[12]
1459,6919	1459,6973	0,0054	4	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
870,3848	870,3849	0,0001	0	59	66	AMGIMNSF	
886,3797	886,3954	0,0157	18	59	66	AMGIMNSF	Oxidation (M)[5]
886,3797	886,3758	-0,0039	-4	59	66	AMGIMNSF	Oxidation (M)[2]
886,3797	886,3954	0,0157	18	59	66	AMGIMNSF	Oxidation (N)[6]
1126,5527	1126,5636	0,0109	10	65	73	SFVNDIFER	
1039,5208	1039,5385	0,0177	17	66	73	FVNDIFER	
934,4992	934,5231	0,0239	26	70	77	IFERIAGE	
1248,6694	1248,6660	-0,0034	-3	70	80	IFERIAGEASR	
1472,7605	1472,7605	0	0	72	84	ERIAGEASRLAHY	
1187,6167	1187,6245	0,0078	7	74	84	IAGEASRLAHY	
1585,8557	1585,8523	-0,0034	-2	74	87	IAGEASRLAHYNKR	
1215,6705	1215,6785	0,008	7	78	87	ASRLAHYNKR	
1990,0101	1990,0554	0,0453	23	78	94	ASRLAHYNKRSTITSRE	Arg->GluSA (R)[10], Carbamyl (R)[16]
1674,8558	1674,8535	-0,0023	-1	81	94	LAHYNKRSTITSRE	Lys->Allysine (K)[6]
1279,8096	1279,8218	0,0122	10	98	109	AVRLLLPGELAK	
1586,9740	1586,9617	-0,0123	-8	98	112	AVRLLLPGELAKHAV	
1803,0487	1803,0527	0,004	2	98	114	AVRLLLPGELAKHAVSE	
896,5563	896,5594	0,0031	3	99	106	VRLLLPGE	
1080,6775	1080,6781	0,0006	1	99	108	VRLLLPGELA	
1236,7311	1236,7025	-0,0286	-23	99	109	VRLLLPGELAK	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1208,7725	1208,7988	0,0263	22	99	109	VRLLLPGELAK	
1515,9369	1515,9318	-0,0051	-3	99	112	VRLLLPGELAKHAV	
1730,9800	1731,0159	0,0359	21	99	114	VRLLLPGELAKHAVSE	Lys->Allysine (K)[11]
1745,9908	1746,0125	0,0217	12	99	114	VRLLLPGELAKHAVSE	Pro->pyro-Glu (P)[6]
1745,9908	1746,0125	0,0217	12	99	114	VRLLLPGELAKHAVSE	Carbonyl (G)[7]
797,4879	797,5065	0,0186	23	100	106	RLLLPGE	
1109,7041	1109,7220	0,0179	16	100	109	RLLLPGELAK	

	1631,9115	1631,9320	0,0205	13	100	114	RLLLPGELAKHAVSE	Lys->Allysine (K)[10]
	1632,9431	1632,9408	-0,0023	-1	100	114	RLLLPGELAKHAVSE	
	953,6030	953,6243	0,0213	22	101	109	LLLPGELAK	
	1476,8420	1476,8359	-0,0061	-4	101	114	LLLPGELAKHAVSE	
	1363,7579	1363,7455	-0,0124	-9	102	114	LLPGELAKHAVSE	
	1685,8229	1685,8598	0,0369	22	104	120	PGELAKHAVSEGTKAVT	His->Asn (H)[7], Lys->Allysine (K)[6], Lys->AminoadipicAcid (K)[14]
	1270,7002	1270,6963	-0,0039	-3	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B	2020,1913	2020,1708	-0,0205	-10	2	21	PEPAKSAPAPKKGSKKAVTK	
Ox_2 - 3h	875,4145	875,4128	-0,0017	-2	35	41	KESYSVY	
	1265,6412	1265,6306	-0,0106	-8	35	44	KESYSVYVYK	
	1605,8887	1605,8616	-0,0271	-17	35	47	KESYSVYVYKVLK	
	1008,5036	1008,4947	-0,0089	-9	37	44	SYSVYVYK	
	1348,7511	1348,7379	-0,0132	-10	37	47	SYSVYVYKVLK	
	2248,2336	2248,2017	-0,0319	-14	39	58	SVYVYKVLKQVHPDTGISSK	
	1587,8893	1587,8569	-0,0324	-20	40	52	VYVYKVLKQVHPD	
	1325,7576	1325,7358	-0,0218	-16	42	52	VYKVLKQVHPD	
	1899,0698	1899,0459	-0,0239	-13	42	58	VYKVLKQVHPDTGISSK	
	1915,0647	1915,0330	-0,0317	-17	42	58	VYKVLKQVHPDTGISSK	Oxidation (K)[17]
	1915,0647	1915,0330	-0,0317	-17	42	58	VYKVLKQVHPDTGISSK	Oxidation (P)[10]
	1915,0647	1915,0330	-0,0317	-17	42	58	VYKVLKQVHPDTGISSK	Oxidation (D)[11]
	2603,3684	2603,3384	-0,0300	-12	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
	2619,3633	2619,3535	-0,0098	-4	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (M)[22]
	2619,3633	2619,3535	-0,0098	-4	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (P)[10]
	2619,3633	2619,3535	-0,0098	-4	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
	2619,3633	2619,3535	-0,0098	-4	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (K)[17]

1800,0015	1799,9800	-0,0215	-12	43	58	YKVLKQVHPDTGISSK	
2341,2366	2341,2214	-0,0152	-6	44	65	KVLKQVHPDTGISSKAMGIMNS	
2488,3049	2488,2959	-0,0090	-4	44	66	KVLKQVHPDTGISSKAMGIMNSF	
2504,3000	2504,2832	-0,0168	-7	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (M)[17]
2504,3000	2504,2832	-0,0168	-7	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[23]
2504,3000	2504,2832	-0,0168	-7	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[15]
2504,3000	2504,2832	-0,0168	-7	44	66	KVLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[8]
1508,8431	1508,8317	-0,0114	-8	45	58	VLKQVHPDTGISSK	
2213,1416	2213,1252	-0,0164	-7	45	65	VLKQVHPDTGISSKAMGIMNS	
2360,2100	2360,2068	-0,0032	-1	45	66	VLKQVHPDTGISSKAMGIMNSF	
2376,2051	2376,1580	-0,0471	-20	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1580	-0,0471	-20	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2376,2051	2376,1580	-0,0471	-20	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[7]
2148,0576	2148,0396	-0,0180	-8	47	66	KQVHPDTGISSKAMGIMNSF	
1443,6970	1443,6846	-0,0124	-9	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6746	-0,0173	-12	53	66	TGISSKAMGIMNSF	Oxidation (F)[14]
1459,6919	1459,7059	0,0140	10	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
1459,6919	1459,7061	0,0142	10	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
870,3848	870,3688	-0,0160	-18	59	66	AMGIMNSF	
886,3797	886,3760	-0,0037	-4	59	66	AMGIMNSF	Oxidation (M)[5]
886,3797	886,3760	-0,0037	-4	59	66	AMGIMNSF	Oxidation (N)[6]
1039,5208	1039,5251	0,0043	4	67	73	FVNDIFER	
892,4523	892,4495	-0,0028	-3	67	73	VNDIFER	
934,4992	934,4976	-0,0016	-2	70	77	IFERIAGE	
1248,6694	1248,6649	-0,0045	-4	70	80	IFERIAGEASR	
1278,6437	1278,6665	0,0228	18	70	80	IFERIAGEASR	Carbonyl (G)[7], Oxidation (R)[11]
1990,0101	1990,0476	0,0375	19	78	94	ASRLAHYNKRSTITSRE	Arg->GluSA (R)[10], Carbamyl (K)[9]
1990,0577	1990,0476	-0,0101	-5	78	94	ASRLAHYNKRSTITSRE	
1989,0261	1988,9939	-0,0322	-16	78	94	ASRLAHYNKRSTITSRE	Lys->Allysine (K)[9]
1675,8875	1675,8853	-0,0022	-1	81	94	LAHYNKRSTITSRE	
1279,8096	1279,8060	-0,0036	-3	98	109	AVRLLLPGELAK	

	1803,0487	1803,0385	-0,0102	-6	98	114	AVRLLLPGELAKHAVSE	
	896,5563	896,5544	-0,0019	-2	99	106	VRLLLPGE	
	1080,6775	1080,6742	-0,0033	-3	99	108	VRLLLPGELA	
	1236,7311	1236,7103	-0,0208	-17	99	108	VRLLLPGELAK	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
	1515,9369	1515,9346	-0,0023	-2	99	112	VRLLLPGELAKHAV	
	1748,0065	1747,9988	-0,0077	-4	99	114	VRLLLPGELAKHAVSE	Oxidation (L)[9]
	1748,0065	1747,9988	-0,0077	-4	99	114	VRLLLPGELAKHAVSE	Oxidation (K)[11]
	1748,0065	1747,9988	-0,0077	-4	99	114	VRLLLPGELAKHAVSE	Oxidation (P)[6]
	1745,9908	1746,0188	0,0280	16	99	114	VRLLLPGELAKHAVSE	Pro->pyro-Glu (P)[6]
	1745,9908	1746,0188	0,0280	16	99	114	VRLLLPGELAKHAVSE	Carbonyl (G)[7]
	1759,9701	1760,0036	0,0335	19	99	114	VRLLLPGELAKHAVSE	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
	1732,0116	1732,0068	-0,0048	-3	99	114	VRLLLPGELAKHAVSE	
	2983,6938	2983,7178	0,0240	8	99	126	VRLLLPGELAKHAVSEGTKAVTKYTSSK	
	1109,7041	1109,7072	0,0031	3	100	109	RLLLPGELAK	
	1632,9431	1632,9370	-0,0061	-4	100	114	RLLLPGELAKHAVSE	
	1476,8420	1476,8323	-0,0097	-7	101	114	LLLPGELAKHAVSE	
	1363,7579	1363,7498	-0,0081	-6	102	114	LLPGELAKHAVSE	
	1270,7002	1270,7065	0,0063	5	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H2B	2020,1913	2020,1844	-0,0069	-3	2	21	PEPAKSAPAPKKGSKKAVTK	
Ox_2 - 24h	1154,6528	1154,6516	-0,0012	-1	4	15	PAKSAPAPKKGS	Oxidation (K)[10]
	1154,6528	1154,6516	-0,0012	-1	4	15	PAKSAPAPKKGS	Oxidation (P)[8]
	875,4145	875,4135	-0,0010	-1	35	41	KESYSVY	
	1137,5463	1137,5355	-0,0108	-9	35	43	KESYSVYVY	
	1265,6412	1265,6337	-0,0075	-6	35	44	KESYSVYVYK	
	1008,5036	1008,4996	-0,0040	-4	37	44	SYSVYVYK	
	2498,3289	2498,2949	-0,0340	-14	37	58	SYSVYVYKVLKQVHPDTGISSK	

2303,1877	2303,1853	-0,0024	-1	38	58	YSVYVYKVLKQVHPDTGISSK	Oxidation (K)[7,10], Oxidation (Y)[1,4,6]
2248,2336	2248,1968	-0,0368	-16	39	58	SVYVYKVLKQVHPDTGISSK	
1011,6237	1011,6118	-0,0119	-12	40	47	VYVYKVLK	
1587,8893	1587,8645	-0,0248	-16	40	52	VYVYKVLKQVHPD	
1946,0746	1946,0463	-0,0283	-15	40	56	VYVYKVLKQVHPDTGIS	
1325,7576	1325,7362	-0,0214	-16	42	52	VYKVLKQVHPD	
1683,9429	1683,9259	-0,0170	-10	42	56	VYKVLKQVHPDTGIS	
1770,9749	1770,9451	-0,0298	-17	42	57	VYKVLKQVHPDTGISS	
1899,0698	1899,0483	-0,0215	-11	42	58	VYKVLKQVHPDTGISSK	
2750,4368	2750,4312	-0,0056	-2	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
2766,4316	2766,3728	-0,0588	-21	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (M)[22]
2766,4316	2766,3728	-0,0588	-21	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[25]
2766,4316	2766,3728	-0,0588	-21	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[17]
2766,4316	2766,3728	-0,0588	-21	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[10]
1226,6892	1226,6698	-0,0194	-16	43	52	YKVLKQVHPD	
1584,8744	1584,8551	-0,0193	-12	43	56	YKVLKQVHPDTGIS	
1800,0015	1799,9757	-0,0258	-14	43	58	YKVLKQVHPDTGISSK	
1421,8112	1421,7987	-0,0125	-9	44	56	KVLKQVHPDTGIS	
1636,9381	1636,9183	-0,0198	-12	44	58	KVLKQVHPDTGISSK	
1293,7162	1293,7040	-0,0122	-9	45	56	VLKQVHPDTGIS	
1380,7482	1380,7140	-0,0342	-25	45	57	VLKQVHPDTGISS	
1508,8431	1508,8389	-0,0042	-3	45	58	VLKQVHPDTGISSK	
1710,9207	1710,9014	-0,0193	-11	45	60	VLKQVHPDTGISSKAM	
1726,9156	1726,8829	-0,0327	-19	45	60	VLKQVHPDTGISSKAM	Oxidation (M)[16]
1726,9156	1726,8829	-0,0327	-19	45	60	VLKQVHPDTGISSKAM	Oxidation (K)[14]
1726,9156	1726,8829	-0,0327	-19	45	60	VLKQVHPDTGISSKAM	Oxidation (D)[8]
1726,9156	1726,8829	-0,0327	-19	45	60	VLKQVHPDTGISSKAM	Oxidation (P)[7]
2360,2100	2360,1975	-0,0125	-5	45	66	VLKQVHPDTGISSKAMGIMNSF	
2392,2000	2392,1562	-0,0438	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Dioxidation (F)[22]
2392,2000	2392,1562	-0,0438	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Dioxidation (M)[19]

2376,2051	2376,1682	-0,0369	-16	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1682	-0,0369	-16	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2376,2051	2376,1682	-0,0369	-16	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[7]
2392,2000	2392,1562	-0,0438	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Dioxidation (P)[7]
2392,2000	2392,1562	-0,0438	-18	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14], Oxidation (P)[7]
2148,0576	2148,0388	-0,0188	-9	47	66	KQVHPDTGISSKAMGIMNSF	
2164,0525	2164,0220	-0,0305	-14	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (M)[17]
2164,0525	2164,0220	-0,0305	-14	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (N)[18]
2164,0525	2164,0220	-0,0305	-14	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (K)[12]
2180,0474	2180,0071	-0,0403	-18	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (F)[20], Oxidation (M)[17]
2180,0474	2180,0071	-0,0403	-18	47	66	KQVHPDTGISSKAMGIMNSF	Dioxidation (F)[20]
2508,1858	2508,1523	-0,0335	-13	47	69	KQVHPDTGISSKAMGIMNSFVND	Oxidation (F)[20], Oxidation (M)[14]
2508,1858	2508,1523	-0,0335	-13	47	69	KQVHPDTGISSKAMGIMNSFVND	Oxidation (D)[23], Oxidation (N)[22]
2508,1858	2508,1523	-0,0335	-13	47	69	KQVHPDTGISSKAMGIMNSFVND	Oxidation (LV)[21], Oxidation (K)[12]
1891,9041	1891,8846	-0,0195	-10	49	66	VHPDTGISSKAMGIMNSF	
1907,8990	1907,8630	-0,0360	-19	49	66	VHPDTGISSKAMGIMNSF	Oxidation (M)[12]
1907,8990	1907,8630	-0,0360	-19	49	66	VHPDTGISSKAMGIMNSF	Oxidation (N)[16]
1907,8990	1907,8630	-0,0360	-19	49	66	VHPDTGISSKAMGIMNSF	Oxidation (K)[10]
2252,0322	2252,0100	-0,0222	-10	49	69	VHPDTGISSKAMGIMNSFVND	Oxidation (F)[18], Oxidation (M)[12]
2144,9839	2145,0363	0,0524	24	51	70	PDTGISSKAMGIMNSFVNDI	Oxidation (F)[16], Oxidation (M)[10,13]
1443,6970	1443,6851	-0,0119	-8	53	66	TGISSKAMGIMNSF	
1475,6869	1475,6595	-0,0274	-19	53	66	TGISSKAMGIMNSF	Oxidation (F)[14], Oxidation (M)[8]
1459,6919	1459,6870	-0,0049	-3	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
1475,6869	1475,6595	-0,0274	-19	53	66	TGISSKAMGIMNSF	Dioxidation (F)[14]
1459,6919	1459,6870	-0,0049	-3	53	66	TGISSKAMGIMNSF	Oxidation (K)[6]
1101,5067	1101,4962	-0,0105	-10	57	66	SKAMGIMNSF	Oxidation (M)[7]
1085,5118	1085,5085	-0,0033	-3	57	66	SKAMGIMNSF	
1101,5067	1101,4962	-0,0105	-10	57	66	SKAMGIMNSF	Oxidation (N)[8]
1117,5017	1117,4939	-0,0078	-7	57	66	SKAMGIMNSF	Oxidation (M)[4,7]
1429,6450	1429,6378	-0,0072	-5	57	69	SKAMGIMNSFVND	Oxidation (M)[4]

1429,6450	1429,6378	-0,0072	-5	57	69	SKAMGIMNSFVND	Oxidation (K)[2]
1014,4747	1014,4739	-0,0008	-1	58	66	KAMGIMNSF	Oxidation (M)[3]
1030,4696	1030,4753	0,0057	6	58	66	KAMGIMNSF	Oxidation (F)[9], Oxidation (M)[3]
1014,4747	1014,4739	-0,0008	-1	58	66	KAMGIMNSF	Oxidation (K)[1]
1014,4747	1014,4739	-0,0008	-1	58	66	KAMGIMNSF	Oxidation (N)[7]
886,3797	886,3696	-0,0101	-11	59	66	AMGIMNSF	Oxidation (M)[5]
870,3848	870,3724	-0,0124	-14	59	66	AMGIMNSF	
886,3797	886,3749	-0,0048	-5	59	66	AMGIMNSF	Oxidation (M)[2]
886,3797	886,3696	-0,0101	-11	59	66	AMGIMNSF	Oxidation (N)[6]
1039,5208	1039,5278	0,0070	7	66	73	FVNDIFER	
934,4992	934,5062	0,0070	7	70	77	IFERIAGE	
1248,6694	1248,6711	0,0017	1	70	80	IFERIAGEASR	
1675,8875	1675,8750	-0,0125	-7	81	94	LAHYNKRSTITSRE	
1622,0000	1621,9960	-0,0040	-2	95	109	IQTAVRLLLPGELAK	
1279,8096	1279,8097	0,0001	0	98	109	AVRLLLPGELAK	
1803,0487	1803,0436	-0,0051	-3	98	114	AVRLLLPGELAKHAVSE	
896,5563	896,5566	0,0003	0	99	106	VRLLLPGE	
1207,7408	1207,7430	0,0022	2	99	109	VRLLLPGELAK	Lys->Allysine (K)[11]
1224,7675	1224,7614	-0,0061	-5	99	109	VRLLLPGELAK	Oxidation (K)[11]
1224,7675	1224,7614	-0,0061	-5	99	109	VRLLLPGELAK	Oxidation (P)[6]
1224,7675	1224,7614	-0,0061	-5	99	109	VRLLLPGELAK	Oxidation (L)[9]
1222,7518	1222,7571	0,0053	4	99	109	VRLLLPGELAK	Pro->pyro-Glu (P)[6]
1222,7518	1222,7571	0,0053	4	99	109	VRLLLPGELAK	Carbonyl (G)[7]
1345,8314	1345,8265	-0,0049	-4	99	110	VRLLLPGELAKH	
1515,9369	1515,9418	0,0049	3	99	112	VRLLLPGELAKHAV	
1732,0116	1732,0149	0,0033	2	99	114	VRLLLPGELAKHAVSE	
1759,9701	1760,0158	0,0457	26	99	114	VRLLLPGELAKHAVSE	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
2983,6938	2983,6951	0,0013	0	99	126	VRLLLPGELAKHAVSEGTKAVTKYTSSK	
1109,7041	1109,7097	0,0056	5	100	109	RLLLPGELAK	
1632,9431	1632,9446	0,0015	1	100	114	RLLLPGELAKHAVSE	
1919,1073	1919,0966	-0,0107	-6	100	117	RLLLPGELAKHAVSEGTK	

953,6030	953,5950	-0,0080	-8	101	109	LLLPGELAK	
1476,8420	1476,8209	-0,0211	-14	101	114	LLLPGELAKHAVSE	
840,5189	840,5239	0,0050	6	102	109	LLPGELAK	
1685,8229	1685,8112	-0,0117	-7	104	120	PGELAKHAVSEGTKAVT	His->Asn (H)[7], Lys->Allysine (K)[6], Lys->AminoadipicAcid (K)[14]
1829,9127	1829,9012	-0,0115	-6	104	121	PGELAKHAVSEGTKAVTK	His->Asn (H)[7], Lys->AminoadipicAcid (K)[14,18]
1270,7002	1270,6992	-0,0010	-1	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B	1892,0963	1892,0879	-0,0084	-4	2	20	PEPAKSAPAPKKGSKKAVT	
Control	2020,1913	2020,1848	-0,0065	-3	2	21	PEPAKSAPAPKKGSKKAVTK	
	2347,3818	2347,3640	-0,0178	-8	2	24	PEPAKSAPAPKKGSKKAVTKAQK	
	2220,2444	2220,2505	0,0061	3	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[9,12]
	2220,2444	2220,2476	0,0032	1	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[8,9]
	1037,4673	1037,4603	-0,0070	-7	35	41	KESYSVY	Glycation (K)[1]
	875,4145	875,4257	0,0112	13	35	41	KESYSVY	
	1137,5463	1137,5483	0,0020	2	35	43	KESYSVYVY	
	1265,6412	1265,6333	-0,0079	-6	35	44	KESYSVYVYK	
	1008,5036	1008,5012	-0,0024	-2	37	44	SYSVYVYK	
	1011,6237	1011,6281	0,0044	4	40	47	VYVYKVLK	
	1325,7576	1325,7511	-0,0065	-5	42	52	VYKVLKQVHPD	
	1770,9749	1770,9445	-0,0304	-17	42	57	VYKVLKQVHPDTGISS	
	1899,0698	1899,0541	-0,0157	-8	42	58	VYKVLKQVHPDTGISSK	
	2213,1416	2213,1169	-0,0247	-11	45	65	VLKQVHPDTGISSKAMGIMNS	
	889,3937	889,4084	0,0147	17	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]
	892,4523	892,4634	0,0111	12	67	73	VNDIFER	
	934,4992	934,5081	0,0089	10	70	77	IFERIAGE	
	1248,6694	1248,6672	-0,0022	-2	70	80	IFERIAGEASR	
	1215,6705	1215,6698	-0,0007	-1	78	87	ASRLAHYNKR	
	1990,0577	1990,0377	-0,0200	-10	78	94	ASRLAHYNKRSTITSRE	
	1546,8448	1546,8336	-0,0112	-7	81	93	LAHYNKRSTITSR	
	1675,8875	1675,8787	-0,0088	-5	81	94	LAHYNKRSTITSRE	
	1794,9960	1794,9799	-0,0161	-9	100	114	RLLLPGELAKHAVSE	Glycation (K)[10]
	1486,7748	1486,7898	0,0150	10	113	126	SEGTKAVTKYTSSK	
	1270,7002	1270,7092	0,0090	7	115	126	GTKAVTKYTSSK	
	1432,7529	1432,7509	-0,0020	-1	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B Ox_1 - 30min	1492,8481	1492,8201	-0,0280	-19	2	16	PEPAKSAPAPKKGSK	
	2220,2444	2220,2451	0,0007	0	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[8,9]
	2590,4297	2590,4600	0,0303	12	5	26	AKSAPAPKKGSKKAVTKAQKKD	Glycation (K)[8,9], Lys->Allysine (K)[21]
	875,4145	875,4310	0,0165	19	35	41	KESYSVY	
	974,4828	974,4832	0,0004	0	35	42	KESYSVYV	
	1137,5463	1137,5465	0,0002	0	35	43	KESYSVYVY	
	1265,6412	1265,6392	-0,0020	-2	35	44	KESYSVYVYK	
	1008,5036	1008,5044	0,0008	1	37	44	SYSVYVYK	
	1098,6558	1098,6539	-0,0019	-2	39	47	SVYVYKVLK	
	1011,6237	1011,6295	0,0058	6	40	47	VYVYKVLK	
	1325,7576	1325,7507	-0,0069	-5	42	52	VYKVLKQVHPD	
	1899,0698	1899,0544	-0,0154	-8	42	58	VYKVLKQVHPDTGISSK	
	889,3937	889,3885	-0,0052	-6	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]
	892,4523	892,4630	0,0107	12	67	73	VNDIFER	
	934,4992	934,5130	0,0138	15	70	77	IFERIAGE	
	1248,6694	1248,6746	0,0052	4	70	80	IFERIAGEASR	
	817,4315	817,4443	0,0128	16	78	84	ASRLAHY	
	1215,6705	1215,6812	0,0107	9	78	87	ASRLAHYNKR	
	1691,8823	1691,8669	-0,0154	-9	81	94	LAHYNKRSTITSRE	Oxidation (Y)[4]
	1691,8823	1691,8669	-0,0154	-9	81	94	LAHYNKRSTITSRE	Oxidation (L)[1]
	1675,8875	1675,8820	-0,0055	-3	81	94	LAHYNKRSTITSRE	
	797,4879	797,5026	0,0147	18	100	106	RLLLPGE	
	1794,9960	1794,9762	-0,0198	-11	100	114	RLLLPGELAKHAVSE	Glycation (K)[10]
	1363,7579	1363,7352	-0,0227	-17	102	114	LLPGELAKHAVSE	
	1486,7748	1486,7693	-0,0055	-4	113	126	SEGTKAVTKYTSSK	
	1432,7529	1432,7418	-0,0111	-8	115	126	GTKAVTKYTSSK	Glycation (K)[3]
	1270,7002	1270,6973	-0,0029	-2	115	126	GTKAVTKYTSSK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B Ox_1 - 1h	1934,0706	1934,0863	0,0157	8	2	20	PEPAKSAPAPKKGSKKAVT	Carbamyl (K)[15], Lys->Allysine (K)[16]
	2262,2927	2262,2561	-0,0366	-16	2	23	PEPAKSAPAPKKGSKKAVTKAQ	Carbamyl (K)[16]
	2591,4150	2591,4670	0,0520	20	2	25	PEPAKSAPAPKKGSKKAVTKAQKK	Carbamyl (K)[15,16], Lys->AminoadipicAcid (K)[23,24]
	2220,2444	2220,2456	0,0012	1	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[8,9], Pro->Pyrrolidinone (P)[5], Proglutamicsealde (P)[7]
	2220,2444	2220,2415	-0,0029	-1	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[8,9]
	2347,3079	2347,3535	0,0456	19	5	24	AKSAPAPKKGSKKAVTKAQK	Glycation (K)[8,9], Lys->Allysine (K)[20]
	2903,6411	2903,6143	-0,0268	-9	5	29	AKSAPAPKKGSKKAVTKAQKKDGKK	Glycation (K)[9,12], Lys->Allysine (K)[25]
	2628,5518	2628,5182	-0,0336	-13	8	30	APAPKKGSKKAVTKAQKKDGKKR	Glycation (K)[5], Oxidation (D)[19]
	875,4145	875,4104	-0,0041	-5	35	41	KESYSVY	
	974,4828	974,4985	0,0157	16	35	42	KESYSVYV	
	1137,5463	1137,5509	0,0046	4	35	43	KESYSVYVY	
	1265,6412	1265,6428	0,0016	1	35	44	KESYSVYVYK	
	1427,6940	1427,6831	-0,0109	-8	35	44	KESYSVYVYK	Glycation (K)[1]
	1008,5036	1008,5089	0,0053	5	37	44	SYSVYVYK	
	1348,7511	1348,7460	-0,0051	-4	37	47	SYSVYVYKVLK	
	970,5607	970,5751	0,0144	15	39	46	SVYVYKVL	
	1098,6558	1098,6542	-0,0016	-1	39	47	SVYVYKVLK	
	1011,6237	1011,6306	0,0069	7	40	47	VYVYKVLK	
	1587,8893	1587,8705	-0,0188	-12	40	52	VYVYKVLKQVHPD	
	2161,2017	2161,1594	-0,0423	-20	40	58	VYVYKVLKQVHPDTGISSK	
	1325,7576	1325,7457	-0,0119	-9	42	52	VYKVLKQVHPD	
	1683,9429	1683,9143	-0,0286	-17	42	56	VYKVLKQVHPDTGIS	
	1770,9749	1770,9543	-0,0206	-12	42	57	VYKVLKQVHPDTGISS	

1899,0698	1899,0442	-0,0256	-13	42	58	VYKVLKQVHPDTGISSK	
2603,3684	2603,3479	-0,0205	-8	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2619,3633	2619,3359	-0,0274	-10	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (M)[19]
2619,3633	2619,3359	-0,0274	-10	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
2619,3633	2619,3359	-0,0274	-10	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (P)[10]
2750,4368	2750,4038	-0,0330	-12	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
1226,6892	1226,6875	-0,0017	-1	43	52	YKVLKQVHPD	
1800,0015	1799,9681	-0,0334	-19	43	58	YKVLKQVHPDTGISSK	
2213,1416	2213,1143	-0,0273	-12	45	65	VLKQVHPDTGISSKAMGIMNS	
2360,2100	2360,1797	-0,0303	-13	45	66	VLKQVHPDTGISSKAMGIMNSF	
2164,0525	2164,0532	0,0007	0	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (F)[20]
2164,0525	2164,0532	0,0007	0	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (K)[12]
1744,8357	1744,8118	-0,0239	-14	49	65	VHPDTGISSKAMGIMNS	
1891,9041	1891,8894	-0,0147	-8	49	66	VHPDTGISSKAMGIMNSF	
1907,8990	1907,8834	-0,0156	-8	49	66	VHPDTGISSKAMGIMNSF	Oxidation (F)[18]
1039,5208	1039,5349	0,0141	14	66	73	FVNDIFER	
934,4992	934,5247	0,0255	27	70	77	IFERIAGE	
1248,6694	1248,6763	0,0069	6	70	80	IFERIAGEASR	
1187,6167	1187,6302	0,0135	11	74	84	IAGEASRLAHY	
1215,6705	1215,6766	0,0061	5	78	87	ASRLAHYNKR	
1674,8558	1674,8309	-0,0249	-15	81	94	LAHYNKRSTITSRE	Lys->Allysine (K)[6]
1675,8875	1675,8666	-0,0209	-12	81	94	LAHYNKRSTITSRE	
1803,0487	1803,0363	-0,0124	-7	98	114	AVRLLLPGELAKHAVSE	
767,5137	767,5206	0,0069	9	99	105	VRLLLPG	
896,5563	896,5798	0,0235	26	99	106	VRLLLPGE	
1009,6404	1009,6527	0,0123	12	99	107	VRLLLPGEL	
1080,6775	1080,6887	0,0112	10	99	108	VRLLLPGELA	
1208,7725	1208,7905	0,0180	15	99	109	VRLLLPGELAK	
1345,8314	1345,8417	0,0103	8	99	110	VRLLLPGELAKH	
1515,9369	1515,9326	-0,0043	-3	99	112	VRLLLPGELAKHAV	
1732,0116	1732,0110	-0,0006	0	99	114	VRLLLPGELAKHAVSE	

	1109,7041	1109,7201	0,0160	14	100	109	RLLLPGELAK	
	1416,8685	1416,8721	0,0036	3	100	112	RLLLPGELAKHAV	
	1588,8792	1588,9038	0,0246	15	100	112	RLLLPGELAKHAV	Glycation (K)[10], His->Asp (H)[11], Oxidation (L)[8], Oxidation (V)[13]
	1631,9115	1631,9071	-0,0044	-3	100	114	RLLLPGELAKHAVSE	Lys->Allysine (K)[10]
	1632,9431	1632,9430	-0,0001	0	100	114	RLLLPGELAKHAVSE	
	1794,9960	1794,9832	-0,0128	-7	100	114	RLLLPGELAKHAVSE	Glycation (K)[10]
	953,6030	953,6136	0,0106	11	101	109	LLLPGELAK	
	1476,8420	1476,8402	-0,0018	-1	101	114	LLLPGELAKHAVSE	
	1416,7329	1416,7423	0,0094	7	104	117	PGELAKHAVSEGTK	His->Asn (H)[7], Oxidation (V)[9]
	1785,8865	1785,9166	0,0301	17	104	119	PGELAKHAVSEGTKAV	Glycation (K)[14], Carbonyl (G)[12], Oxidation (V)[16]
	1486,7748	1486,7594	-0,0154	-10	113	126	SEGTKAVTKYTSSK	
	1270,7002	1270,7086	0,0084	7	115	126	GTKAVTKYTSSK	
	1432,7529	1432,7600	0,0071	5	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B Ox_1 - 3h	1492,8481	1492,8313	-0,0168	-11	2	16	PEPAKSAPAPKKGSK	
	1892,0963	1892,0789	-0,0174	-9	2	20	PEPAKSAPAPKKGSKKAVT	
	2262,2075	2262,1505	-0,0570	-25	2	20	PEPAKSAPAPKKGSKKAVT	Glycation (K)[11,12], Oxidation (K)[15], Proglutamicsealde (P)[10]
	2036,1862	2036,1691	-0,0171	-8	2	21	PEPAKSAPAPKKGSKKAVTK	Oxidation (K)[5]
	3360,0073	3359,9654	-0,0419	-12	2	32	PEPAKSAPAPKKGSKKAVTKAQKKDGKKRKR	Oxidation (K)[16]
	2220,2444	2220,2407	-0,0037	-2	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[8,9]
	2591,4614	2591,4487	-0,0127	-5	5	26	AKSAPAPKKGSKKAVTKAQKKD	Glycation (K)[9,12]
	3060,7737	3060,7285	-0,0452	-15	5	30	AKSAPAPKKGSKKAVTKAQKKDGKKR	Glycation (K)[8,9]
	3359,9443	3359,9654	0,0211	6	6	32	KSAPAPKKGSKKAVTKAQKKDGKKRKR	Glycation (K)[7,8], Carbamyl (K)[11,12]

2334,2397	2334,1841	-0,0556	-24	8	26	APAPKKGSKKAVTKAQKKD	Glycation (K)[5,9], Carbonyl (G)[7], Lys->AminoadipicAcid (K)[10]
875,4145	875,4270	0,0125	14	35	41	KESYSVY	
1037,4673	1037,4520	-0,0153	-15	35	41	KESYSVY	Glycation (K)[1]
974,4828	974,4799	-0,0029	-3	35	42	KESYSVYV	
1137,5463	1137,5264	-0,0199	-17	35	43	KESYSVYVY	
1265,6412	1265,6340	-0,0072	-6	35	44	KESYSVYVYK	
1427,6940	1427,6581	-0,0359	-25	35	44	KESYSVYVYK	Glycation (K)[1]
1605,8887	1605,8623	-0,0264	-16	35	47	KESYSVYVYKVLK	
1008,5036	1008,5105	0,0069	7	37	44	SYSVYVYK	
1220,6561	1220,6398	-0,0163	-13	37	46	SYSVYVYKVL	
1348,7511	1348,7336	-0,0175	-13	37	47	SYSVYVYKVLK	
1098,6558	1098,6289	-0,0269	-24	39	47	SVYVYKVLK	
1674,9214	1674,9174	-0,0040	-2	39	52	SVYVYKVLKQVHPD	
2248,2336	2248,1912	-0,0424	-19	39	58	SVYVYKVLKQVHPDTGISSK	
1011,6237	1011,6277	0,0040	4	40	47	VYVYKVLK	
1587,8893	1587,8747	-0,0146	-9	40	52	VYVYKVLKQVHPD	
2161,2017	2161,1921	-0,0096	-4	40	58	VYVYKVLKQVHPDTGISSK	
1325,7576	1325,7616	0,0040	3	42	52	VYKVLKQVHPD	
1683,9429	1683,9223	-0,0206	-12	42	56	VYKVLKQVHPDTGIS	
1770,9749	1770,9614	-0,0135	-8	42	57	VYKVLKQVHPDTGISS	
1898,0382	1898,0513	0,0131	7	42	58	VYKVLKQVHPDTGISSK	Lys->Allysine (K)[17]
1899,0698	1899,0751	0,0053	3	42	58	VYKVLKQVHPDTGISSK	
1915,0647	1915,0763	0,0116	6	42	58	VYKVLKQVHPDTGISSK	Oxidation (K)[17]
1915,0647	1915,0763	0,0116	6	42	58	VYKVLKQVHPDTGISSK	Oxidation (D)[11]
1915,0647	1915,0763	0,0116	6	42	58	VYKVLKQVHPDTGISSK	Oxidation (P)[10]
1915,0647	1915,0763	0,0116	6	42	58	VYKVLKQVHPDTGISSK	Oxidation (V)[1]
1915,0647	1915,0763	0,0116	6	42	58	VYKVLKQVHPDTGISSK	Oxidation (Y)[2]
2101,1475	2101,1452	-0,0023	-1	42	60	VYKVLKQVHPDTGISSKAM	
2117,1423	2117,1296	-0,0127	-6	42	60	VYKVLKQVHPDTGISSKAM	Oxidation (M)[19]
2117,1423	2117,1296	-0,0127	-6	42	60	VYKVLKQVHPDTGISSKAM	Oxidation (K)[17]

2117,1423	2117,1296	-0,0127	-6	42	60	VYKVLKQVHPDTGISSKAM	Oxidation (P)[10]
2117,1423	2117,1296	-0,0127	-6	42	60	VYKVLKQVHPDTGISSKAM	Oxidation (D)[11]
2271,2529	2271,2363	-0,0166	-7	42	62	VYKVLKQVHPDTGISSKAMGI	
2603,3684	2603,3134	-0,0550	-21	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2619,3633	2619,3690	0,0057	2	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
1226,6892	1226,7006	0,0114	9	43	52	YKVLKQVHPD	
1800,0015	1799,9915	-0,0100	-6	43	58	YKVLKQVHPDTGISSK	
1508,8431	1508,8389	-0,0042	-3	45	58	VLKQVHPDTGISSK	
2213,1416	2213,1374	-0,0042	-2	45	65	VLKQVHPDTGISSKAMGIMNS	
2360,2100	2360,1906	-0,0194	-8	45	66	VLKQVHPDTGISSKAMGIMNSF	
2392,2000	2392,1511	-0,0489	-20	45	66	VLKQVHPDTGISSKAMGIMNSF	Dioxidation (F)[22]
2392,2000	2392,1511	-0,0489	-20	45	66	VLKQVHPDTGISSKAMGIMNSF	Dioxidation (M)[19]
2148,0576	2148,0764	0,0188	9	47	66	KQVHPDTGISSKAMGIMNSF	
1891,9041	1891,9103	0,0062	3	49	66	VHPDTGISSKAMGIMNSF	
1443,6970	1443,7038	0,0068	5	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6803	-0,0116	-8	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
1459,6919	1459,6803	-0,0116	-8	53	66	TGISSKAMGIMNSF	Oxidation (F)[14]
1085,5118	1085,5044	-0,0074	-7	57	66	SKAMGIMNSF	
870,3848	870,3865	0,0017	2	59	66	AMGIMNSF	
889,3937	889,4006	0,0069	8	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]
1126,5527	1126,5410	-0,0117	-10	65	73	SFVNDIFER	
754,3770	754,3889	0,0119	16	66	71	FVNDIF	
1039,5208	1039,5222	0,0014	1	66	73	FVNDIFER	
934,4992	934,5068	0,0076	8	70	77	IFERIAGE	
1248,6694	1248,6823	0,0129	10	70	80	IFERIAGEASR	
1135,5854	1135,6075	0,0221	19	71	80	FERIAGEASR	
1187,6167	1187,5840	-0,0327	-28	74	84	IAGEASRLAHY	
1429,7546	1429,7570	0,0024	2	74	86	IAGEASRLAHYNK	
1215,6705	1215,6777	0,0072	6	78	87	ASRLAHYNKR	
1674,8558	1674,8505	-0,0053	-3	81	94	LAHYNKRSTITSRE	Lys->Allysine (K)[6]
1675,8875	1675,8819	-0,0056	-3	81	94	LAHYNKRSTITSRE	

1751,0425	1751,0221	-0,0204	-12	94	109	EIQTAVRLLLPGELAK	
1309,7838	1309,7470	-0,0368	-28	95	106	IQTAVRLLLPGE	
1279,8096	1279,8146	0,0050	4	98	109	AVRLLLPGELAK	
1586,9740	1586,9980	0,0240	15	98	112	AVRLLLPGELAKHAV	
1803,0487	1803,0834	0,0347	19	98	114	AVRLLLPGELAKHAVSE	
767,5137	767,5014	-0,0123	-16	99	105	VRLLLPG	
896,5563	896,5801	0,0238	27	99	106	VRLLLPGE	
1009,6404	1009,6432	0,0028	3	99	107	VRLLLPGEL	
1236,7311	1236,7202	-0,0109	-9	99	109	VRLLLPGELAK	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1515,9369	1515,8939	-0,0430	-28	99	112	VRLLLPGELAKHAV	
1730,9800	1730,9846	0,0046	3	99	114	VRLLLPGELAKHAVSE	Lys->Allysine (K)[11]
1732,0116	1731,9927	-0,0189	-11	99	114	VRLLLPGELAKHAVSE	
1894,0643	1894,0664	0,0021	1	99	114	VRLLLPGELAKHAVSE	Glycation (K)[11]
1108,6725	1108,6519	-0,0206	-19	100	109	RLLLPGELAK	Lys->Allysine (K)[10]
1109,7041	1109,6951	-0,0090	-8	100	109	RLLLPGELAK	
1137,6627	1137,6791	0,0164	14	100	109	RLLLPGELAK	Carbonyl (G)[6], Pro->pyro-Glu (P)[5]
1416,8685	1416,8881	0,0196	14	100	112	RLLLPGELAKHAV	
1631,9115	1631,9146	0,0031	2	100	114	RLLLPGELAKHAVSE	Lys->Allysine (K)[10]
1632,9431	1632,9539	0,0108	7	100	114	RLLLPGELAKHAVSE	
1646,9224	1646,9477	0,0253	15	100	114	RLLLPGELAKHAVSE	Pro->pyro-Glu (P)[5]
1646,9225	1646,9477	0,0252	15	100	114	RLLLPGELAKHAVSE	Carbonyl (G)[6]
1476,8420	1476,8280	-0,0140	-9	101	114	LLLPGELAKHAVSE	
1486,7748	1486,7639	-0,0109	-7	113	126	SEGTKAVTKYTSSK	
1270,7002	1270,6973	-0,0029	-2	115	126	GTKAVTKYTSSK	
1432,7529	1432,7587	0,0058	4	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B	2062,1655	2062,1445	-0,0210	-10	2	21	PEPAKSAPAPKKGSKKAVTK	Carbamyl (K)[16], Lys->Allysine (K)[20]

Ox_1 - 24h	2262,2075	2262,1762	-0,0313	-14	2	20	PEPAKSAPAPKKGSKKAVT	Glycation (K)[11,12], Oxidation (K)[15], Proglutamicsealde (P)[10]
	2591,4614	2591,4439	-0,0175	-7	5	26	AKSAPAPKKGSKKAVTKAQKKD	Glycation (K)[9,12]
	3060,7737	3060,7550	-0,0187	-6	5	30	AKSAPAPKKGSKKAVTKAQKKDGKKR	Glycation (K)[8,9]
	3359,9443	3359,9166	-0,0277	-8	6	32	KSAPAPKKGSKKAVTKAQKKDGKKRKR	Glycation (K)[7,8], Carbamyl (K)[11,12]
	2334,2397	2334,2087	-0,0310	-13	8	26	APAPKKGSKKAVTKAQKKD	Glycation (K)[5,9], Carbonyl (G)[7], Lys->AminoadipicAcid (K)[10]
	875,4145	875,4067	-0,0078	-9	35	41	KESYSVY	
	1137,5463	1137,5796	0,0333	29	35	43	KESYSVYVY	
	1265,6412	1265,6372	-0,0040	-3	35	44	KESYSVYVYK	
	1220,6561	1220,6611	0,0050	4	37	46	SYSVYVYKVL	
	1925,0167	1924,9923	-0,0244	-13	37	52	SYSVYVYKVLKQVHPD	
	2498,3289	2498,3214	-0,0075	-3	37	58	SYSVYVYKVLKQVHPDTGISSK	
	1674,9214	1674,9514	0,0300	18	39	52	SVYVYKVLKQVHPD	
	2248,2336	2248,2528	0,0192	9	39	58	SVYVYKVLKQVHPDTGISSK	
	1587,8893	1587,8748	-0,0145	-9	40	52	VYVYKVLKQVHPD	
	2161,2017	2161,1901	-0,0116	-5	40	58	VYVYKVLKQVHPDTGISSK	
	1325,7576	1325,7504	-0,0072	-5	42	52	VYKVLKQVHPD	
	1683,9429	1683,9189	-0,0240	-14	42	56	VYKVLKQVHPDTGIS	
	1770,9749	1770,9800	0,0051	3	42	57	VYKVLKQVHPDTGISS	
	1899,0698	1899,0974	0,0276	15	42	58	VYKVLKQVHPDTGISSK	
	2101,1475	2101,0964	-0,0511	-24	42	60	VYKVLKQVHPDTGISSKAM	
	2603,3684	2603,3515	-0,0169	-6	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
	2766,4316	2766,3930	-0,0386	-14	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[25]
	2766,4316	2766,3930	-0,0386	-14	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[17]
	2766,4316	2766,3930	-0,0386	-14	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[10]
	2750,4368	2750,4025	-0,0343	-12	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
	1800,0015	1799,9906	-0,0109	-6	43	58	YKVLKQVHPDTGISSK	
	1421,8112	1421,7930	-0,0182	-13	44	56	KVLKQVHPDTGIS	
	1293,7162	1293,6930	-0,0232	-18	45	56	VLKQVHPDTGIS	

1380,7482	1380,7476	-0,0006	0	45	57	VLKQVHPDTGISS	
1508,8431	1508,8335	-0,0096	-6	45	58	VLKQVHPDTGISSK	
1507,8115	1507,7865	-0,0250	-17	45	58	VLKQVHPDTGISSK	Lys->Allysine (K)[14]
2213,1416	2213,1557	0,0141	6	45	65	VLKQVHPDTGISSKAMGIMNS	
2376,2051	2376,1786	-0,0265	-11	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (N)[20]
2376,2051	2376,1786	-0,0265	-11	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1786	-0,0265	-11	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2360,2100	2360,2226	0,0126	5	45	66	VLKQVHPDTGISSKAMGIMNSF	
1799,9142	1799,9027	-0,0115	-6	47	63	KQVHPDTGISSKAMGIM	
2164,0525	2163,9953	-0,0572	-26	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (F)[20]
2148,0576	2148,0822	0,0246	11	47	66	KQVHPDTGISSKAMGIMNSF	
2035,9575	2035,9454	-0,0121	-6	48	66	QVHPDTGISSKAMGIMNSF	Oxidation (F)[19]
1891,9041	1891,8849	-0,0192	-10	49	66	VHPDTGISSKAMGIMNSF	
1443,6970	1443,6888	-0,0082	-6	53	66	TGISSKAMGIMNSF	
1101,5067	1101,5053	-0,0014	-1	57	66	SKAMGIMNSF	Oxidation (M)[7]
1101,5067	1101,5053	-0,0014	-1	57	66	SKAMGIMNSF	Oxidation (N)[8]
1085,5118	1085,5075	-0,0043	-4	57	66	SKAMGIMNSF	
998,4797	998,4929	0,0132	13	58	66	KAMGIMNSF	
870,3848	870,4027	0,0179	21	59	66	AMGIMNSF	
754,3770	754,3796	0,0026	3	66	71	FVNDIF	
1039,5208	1039,5333	0,0125	12	66	73	FVNDIFER	
892,4523	892,4544	0,0021	2	67	73	VNDIFER	
1262,6376	1262,6503	0,0127	10	67	77	VNDIFERIAGE	
964,4734	964,4976	0,0242	25	70	77	IFERIAGE	Carbonyl (G)[7], Oxidation (R)[4]
934,4992	934,5052	0,0060	6	70	77	IFERIAGE	
1248,6694	1248,6769	0,0075	6	70	80	IFERIAGEASR	
1135,5854	1135,6097	0,0243	21	71	80	FERIAGEASR	
1675,8875	1675,8586	-0,0289	-17	81	94	LAHYNKRSTITSRE	
1675,8398	1675,8445	0,0047	3	81	94	LAHYNKRSTITSRE	Arg->GluSA (R)[7], Carbamyl (K)[6]
1751,0425	1751,0393	-0,0032	-2	94	109	EIQTAVRLLLPGELAK	
1309,7838	1309,8002	0,0164	13	95	106	IQTAVRLLLPGE	

1422,8679	1422,8610	-0,0069	-5	95	107	IQTAVRLLLPGEL	
1622,0000	1621,9915	-0,0085	-5	95	109	IQTAVRLLLPGELAK	
2145,2390	2145,1877	-0,0513	-24	95	114	IQTAVRLLLPGELAKHAVSE	
1919,1073	1919,0625	-0,0448	-23	96	113	QTAVRLLLPGELAKHAVS	Oxidation (P)[9]
1919,1072	1919,0625	-0,0447	-23	96	113	QTAVRLLLPGELAKHAVS	Oxidation (L)[8]
1803,0487	1803,0361	-0,0126	-7	98	114	AVRLLLPGELAKHAVSE	
767,5137	767,5195	0,0058	8	99	105	VRLLLPG	
896,5563	896,5453	-0,0110	-12	99	106	VRLLLPGE	
1037,5990	1037,6198	0,0208	20	99	107	VRLLLPGEL	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1009,6404	1009,6373	-0,0031	-3	99	107	VRLLLPGEL	
1236,7311	1236,7271	-0,0040	-3	99	109	VRLLLPGELAK	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1222,7518	1222,7618	0,0100	8	99	109	VRLLLPGELAK	Pro->pyro-Glu (P)[6]
1207,7408	1207,7543	0,0135	11	99	109	VRLLLPGELAK	Lys->Allysine (K)[11]
1515,9369	1515,9304	-0,0065	-4	99	112	VRLLLPGELAKHAV	
1732,0116	1732,0013	-0,0103	-6	99	114	VRLLLPGELAKHAVSE	
1730,9800	1731,0027	0,0227	13	99	114	VRLLLPGELAKHAVSE	Lys->Allysine (K)[11]
1137,6627	1137,6899	0,0272	24	100	109	RLLLPGELAK	Carbonyl (G)[6], Pro->pyro-Glu (P)[5]
1109,7041	1109,7095	0,0054	5	100	109	RLLLPGELAK	
1108,6725	1108,6512	-0,0213	-19	100	109	RLLLPGELAK	Lys->Allysine (K)[10]
1632,9431	1632,9305	-0,0126	-8	100	114	RLLLPGELAKHAVSE	
1794,9960	1794,9903	-0,0057	-3	100	114	RLLLPGELAKHAVSE	Glycation (K)[10]
1476,8420	1476,8307	-0,0113	-8	101	114	LLLPGELAKHAVSE	
871,4771	871,4847	0,0076	9	102	109	LLPGELAK	Oxidation (L)[2], Lys->AminoadipicAcid (K)[8]
1631,7759	1631,7989	0,0230	14	104	117	PGELAKHAVSEGTK	Glycation (K)[14], Carbonyl (G)[2], Oxidation (K)[6], Oxidation (P)[1]
1486,7748	1486,7841	0,0093	6	113	126	SEGTKAVTKYTSSK	
1270,7002	1270,6913	-0,0089	-7	115	126	GTKAVTKYTSSK	
1432,7529	1432,7499	-0,0030	-2	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B Ox_2 - 30min	2020,1913	2020,1685	-0,0228	-11	2	21	PEPAKSAPAPKKGSKKAVTK	
	2347,3079	2347,3452	0,0373	16	5	24	AKSAPAPKKGSKKAVTKAQK	Glycation (K)[8,9], Lys->Allysine (K)[20]
	2590,4297	2590,4556	0,0259	10	5	26	AKSAPAPKKGSKKAVTKAQKKD	Glycation (K)[8,9], Lys->Allysine (K)[21]
	875,4145	875,4288	0,0143	16	35	41	KESYSVY	
	1137,5463	1137,5492	0,0029	3	35	43	KESYSVYVY	
	1427,6940	1427,6750	-0,0190	-13	35	44	KESYSVYVYK	Glycation (K)[1]
	1265,6412	1265,6401	-0,0011	-1	35	44	KESYSVYVYK	
	1008,5036	1008,5087	0,0051	5	37	44	SYSVYVYK	
	1098,6558	1098,6553	-0,0005	0	39	47	SVYVYKVLK	
	1011,6237	1011,6216	-0,0021	-2	40	47	VYVYKVLK	
	1325,7576	1325,7479	-0,0097	-7	42	52	VYKVLKQVHPD	
	2213,1416	2213,1138	-0,0278	-13	45	65	VLKQVHPDTGISSKAMGIMNS	
	886,3797	886,3847	0,0050	6	59	66	AMGIMNSF	Oxidation (M)[5]
	886,3797	886,3847	0,0050	6	59	66	AMGIMNSF	Oxidation (N)[6]
	889,3937	889,4021	0,0084	9	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]
	892,4523	892,4703	0,0180	20	67	73	VNDIFER	
	934,4992	934,5189	0,0197	21	70	77	IFERIAGE	
	1248,6694	1248,6730	0,0036	3	70	80	IFERIAGEASR	
	1059,5695	1059,5829	0,0134	13	78	86	ASRLAHYNK	
	1058,5378	1058,5364	-0,0014	-1	78	86	ASRLAHYNK	Lys->Allysine (K)[9]
	1231,6654	1231,6691	0,0037	3	78	87	ASRLAHYNKR	Oxidation (R)[3]
	1215,6705	1215,6847	0,0142	12	78	87	ASRLAHYNKR	
	1214,6389	1214,6392	0,0003	0	78	87	ASRLAHYNKR	Lys->Allysine (K)[9]
	1845,8824	1845,9113	0,0289	16	81	94	LAHYNKRSTITSRE	His->Asn (H)[3], Lys->AminoadipicAcid (K)[6], Oxidation (Y)[4]
	1675,8875	1675,8810	-0,0065	-4	81	94	LAHYNKRSTITSRE	
	1674,8558	1674,8644	0,0086	5	81	94	LAHYNKRSTITSRE	Lys->Allysine (K)[6]

	2057,1235	2057,1616	0,0381	19	96	114	QTAVRLLLPGELAKHAVSE	His->Asn (H)[15], Oxidation (L)[6], Oxidation (V)[4,17]
	797,4879	797,5046	0,0167	21	100	106	RLLLPGE	
	1109,7041	1109,7161	0,0120	11	100	109	RLLLPGELAK	
	1794,9960	1794,9917	-0,0043	-2	100	114	RLLLPGELAKHAVSE	Glycation (K)[10]
	1271,6115	1271,6226	0,0111	9	104	116	PGELAKHAVSEGT	His->Asn (H)[7], Lys->Allysine (K)[6]
	1486,7748	1486,7649	-0,0099	-7	113	126	SEGTKAVTKYTSSK	
	1270,7002	1270,7050	0,0048	4	115	126	GTKAVTKYTSSK	
	1269,6686	1269,6544	-0,0142	-11	115	126	GTKAVTKYTSSK	Lys->Allysine (K)[12]
	1432,7529	1432,7535	0,0006	0	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B Ox_2 - 1h	996,4996	996,5091	0,0095	10	2	11	PEPAKSAPAP	Oxidation (P)[3,8]
	1832,9487	1832,9619	0,0132	7	2	16	PEPAKSAPAPKKGSK	Glycation (K)[11,12], Oxidation (P)[10]
	2232,1968	2232,2393	0,0425	19	2	20	PEPAKSAPAPKKGSKKAVT	Glycation (K)[11,12], Oxidation (P)[10]
	2220,2332	2220,2383	0,0051	2	4	21	PAKSAPAPKKGSKKAVTK	Glycation (K)[9,10,13], Pro->Pyrrolidinone (P)[6,8]
	3059,7534	3059,7217	-0,0317	-10	4	30	PAKSAPAPKKGSKKAVTKAQKKDGKKR	Glycation (K)[9], Dioxidation (P)[6,8]
	2220,2444	2220,2383	-0,0061	-3	5	23	AKSAPAPKKGSKKAVTKAQ	Glycation (K)[8,9]
	2347,3079	2347,3289	0,0210	9	5	24	AKSAPAPKKGSKKAVTKAQK	Glycation (K)[8,9], Lys->Allysine (K)[20]
	2590,4297	2590,4343	0,0046	2	5	26	AKSAPAPKKGSKKAVTKAQKKD	Glycation (K)[8,9], Lys->Allysine (K)[21]
	875,4145	875,4386	0,0241	28	35	41	KESYSVY	
	1137,5463	1137,5535	0,0072	6	35	43	KESYSVYVY	
	1265,6412	1265,6375	-0,0037	-3	35	44	KESYSVYVYK	
	1008,5036	1008,5105	0,0069	7	37	44	SYSVYVYK	
	1220,6561	1220,6470	-0,0091	-7	37	46	SYSVYVYKVL	
	1348,7511	1348,7474	-0,0037	-3	37	47	SYSVYVYKVLK	
	970,5607	970,5716	0,0109	11	39	46	SVYVYKVL	

1098,6558	1098,6572	0,0014	1	39	47	SVYVYKVLK	
2248,2336	2248,2153	-0,0183	-8	39	58	SVYVYKVLKQVHPDTGISSK	
1011,6237	1011,6246	0,0009	1	40	47	VYVYKVLK	
1587,8893	1587,8726	-0,0167	-11	40	52	VYVYKVLKQVHPD	
1325,7576	1325,7539	-0,0037	-3	42	52	VYKVLKQVHPD	
1683,9429	1683,9291	-0,0138	-8	42	56	VYKVLKQVHPDTGIS	
1770,9749	1770,9369	-0,0380	-21	42	57	VYKVLKQVHPDTGISS	
1899,0698	1899,0466	-0,0232	-12	42	58	VYKVLKQVHPDTGISSK	
1915,0647	1915,0483	-0,0164	-9	42	58	VYKVLKQVHPDTGISSK	Oxidation (K)[17]
2101,1475	2101,1233	-0,0242	-12	42	60	VYKVLKQVHPDTGISSKAM	
2603,3684	2603,3125	-0,0559	-21	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2619,3633	2619,3298	-0,0335	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
2619,3633	2619,3298	-0,0335	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (M)[22]
2619,3633	2619,3298	-0,0335	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (P)[10]
2619,3633	2619,3298	-0,0335	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (K)[17]
1800,0015	1799,9666	-0,0349	-19	43	58	YKVLKQVHPDTGISSK	
1293,7162	1293,7181	0,0019	1	45	56	VLKQVHPDTGIS	
1508,8431	1508,8398	-0,0033	-2	45	58	VLKQVHPDTGISSK	
2213,1416	2213,1099	-0,0317	-14	45	65	VLKQVHPDTGISSKAMGIMNS	
2148,0576	2148,0247	-0,0329	-15	47	66	KQVHPDTGISSKAMGIMNSF	
1744,8357	1744,8097	-0,0260	-15	49	65	VHPDTGISSKAMGIMNS	
1891,9041	1891,8932	-0,0109	-6	49	66	VHPDTGISSKAMGIMNSF	
1907,8990	1907,8827	-0,0163	-9	49	66	VHPDTGISSKAMGIMNSF	Oxidation (N)[16]
1907,8990	1907,8827	-0,0163	-9	49	66	VHPDTGISSKAMGIMNSF	Oxidation (M)[15]
1443,6970	1443,6844	-0,0126	-9	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6821	-0,0098	-7	53	66	TGISSKAMGIMNSF	Oxidation (M)[11]
1459,6919	1459,6821	-0,0098	-7	53	66	TGISSKAMGIMNSF	Oxidation (N)[12]
1459,6919	1459,7114	0,0195	13	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
1459,6919	1459,7114	0,0195	13	53	66	TGISSKAMGIMNSF	Oxidation (K)[6]
1085,5118	1085,5160	0,0042	4	57	66	SKAMGIMNSF	
870,3848	870,3804	-0,0044	-5	59	66	AMGIMNSF	

886,3797	886,3919	0,0122	14	59	66	AMGIMNSF	Oxidation (M)[5]
1198,5231	1198,5140	-0,0091	-8	59	69	AMGIMNSFVND	
889,3937	889,4139	0,0202	23	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]
1039,5208	1039,5348	0,0140	13	66	73	FVNDIFER	
892,4523	892,4728	0,0205	23	67	73	VNDIFER	
934,4992	934,5092	0,0100	11	70	77	IFERIAGE	
1248,6694	1248,6757	0,0063	5	70	80	IFERIAGEASR	
1215,6705	1215,6848	0,0143	12	78	87	ASRLAHYNKR	
1231,6654	1231,6709	0,0055	4	78	87	ASRLAHYNKR	Oxidation (R)[3]
1846,9518	1846,9489	-0,0029	-2	80	94	RLAHYNKRSTITSRE	Lys->AminoadipicAcid (K)[7]
1675,8875	1675,8647	-0,0228	-14	81	94	LAHYNKRSTITSRE	
1845,8824	1845,8651	-0,0173	-9	81	94	LAHYNKRSTITSRE	His->Asn (H)[3], Lys->AminoadipicAcid (K)[6], Oxidation (Y)[4]
1309,7838	1309,7906	0,0068	5	95	106	IQTAVRLLLPGE	
2057,1235	2057,1499	0,0264	13	96	114	QTAVRLLLPGELAKHAVSE	His->Asn (H)[15], Oxidation (L)[6], Oxidation (V)[4,17]
1279,8096	1279,8119	0,0023	2	98	109	AVRLLLPGELAK	
1803,0487	1803,0394	-0,0093	-5	98	114	AVRLLLPGELAKHAVSE	
767,5137	767,5181	0,0044	6	99	105	VRLLLPG	
896,5563	896,5786	0,0223	25	99	106	VRLLLPGE	
1037,5990	1037,5819	-0,0171	-16	99	107	VRLLLPGEL	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1208,7725	1208,7894	0,0169	14	99	109	VRLLLPGELAK	
1236,7311	1236,7555	0,0244	20	99	109	VRLLLPGELAK	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1345,8314	1345,8344	0,0030	2	99	110	VRLLLPGELAKH	
1515,9369	1515,9313	-0,0056	-4	99	112	VRLLLPGELAKHAV	
1730,9800	1730,9966	0,0166	10	99	114	VRLLLPGELAKHAVSE	Lys->Allysine (K)[11]
1732,0116	1732,0155	0,0039	2	99	114	VRLLLPGELAKHAVSE	
1109,7041	1109,7213	0,0172	15	100	109	RLLLPGELAK	
1271,7570	1271,7556	-0,0014	-1	100	109	RLLLPGELAK	Glycation (K)[10]
1416,8685	1416,8701	0,0016	1	100	112	RLLLPGELAKHAV	

	1624,8905	1624,8802	-0,0103	-6	100	114	RLLLPGELAKHAVSE	His->Asn (H)[11], Lys->AminoadipicAcid (K)[10]
	1631,9115	1631,8994	-0,0121	-7	100	114	RLLLPGELAKHAVSE	Lys->Allysine (K)[10]
	1632,9431	1632,9498	0,0067	4	100	114	RLLLPGELAKHAVSE	
	953,6030	953,6201	0,0171	18	101	109	LLLPGELAK	
	1476,8420	1476,8381	-0,0039	-3	101	114	LLLPGELAKHAVSE	
	1363,7579	1363,7433	-0,0146	-11	102	114	LLPGELAKHAVSE	
	1486,7748	1486,7612	-0,0136	-9	113	126	SEGTKAVTKYTSSK	
	1269,6686	1269,6519	-0,0167	-13	115	126	GTKAVTKYTSSK	Lys->Allysine (K)[12]
	1270,7002	1270,6948	-0,0054	-4	115	126	GTKAVTKYTSSK	
	1432,7529	1432,7576	0,0047	3	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B Ox_2 - 3h	1934,0706	1934,0588	-0,0118	-6	2	20	PEPAKSAPAPKKGSKKAVT	Carbamyl (K)[15], Lys->Allysine (K)[16]
	2062,1655	2062,1528	-0,0127	-6	2	21	PEPAKSAPAPKKGSKKAVTK	Carbamyl (K)[15], Lys->Allysine (K)[16]
	2376,2896	2376,2666	-0,0230	-10	2	21	PEPAKSAPAPKKGSKKAVTK	Glycation (K)[11,12], Oxidation (P)[8,10], Pro->Pyrrolidinone (P)[3], Proglutamicsealde (P)[1]
	2262,1975	2262,2222	0,0247	11	2	23	PEPAKSAPAPKKGSKKAVTKAQ	Lys->Allysine (K)[12,15], Lys->AminoadipicAcid (K)[16], Proglutamicsealde (P)[10]
	2262,2927	2262,2822	-0,0105	-5	2	23	PEPAKSAPAPKKGSKKAVTKAQ	Carbamyl (K)[16]
	2219,2129	2219,2202	0,0073	3	4	22	PAKSAPAPKKGSKKAVTKA	Glycation (K)[9,10], Oxidation (K)[14], Pro->pyro-Glu (P)[6]
	3343,9495	3343,8928	-0,0567	-17	4	32	PAKSAPAPKKGSKKAVTKAQKKDGKKRKR	Glycation (K)[14], Oxidation (K)[21,22], Oxidation (P)[6,8]
	2171,2031	2171,2400	0,0369	17	8	26	APAPKKGSKKAVTKAQKKD	Glycation (K)[6], Carbonyl (G)[7], Pro->pyro-Glu (P)[4]

875,4145	875,4258	0,0113	13	35	41	KESYSVY	
1137,5463	1137,5709	0,0246	22	35	43	KESYSVYVY	
1265,6412	1265,6385	-0,0027	-2	35	44	KESYSVYVYK	
1008,5036	1008,5088	0,0052	5	37	44	SYSVYVYK	
1220,6561	1220,6416	-0,0145	-12	37	46	SYSVYVYKVL	
1348,7511	1348,7460	-0,0051	-4	37	47	SYSVYVYKVLK	
2498,3289	2498,2838	-0,0451	-18	37	58	SYSVYVYKVLKQVHPDTGISSK	
1098,6558	1098,6543	-0,0015	-1	39	47	SVYVYKVLK	
2248,2336	2248,1868	-0,0468	-21	39	58	SVYVYKVLKQVHPDTGISSK	
1011,6237	1011,6315	0,0078	8	40	47	VYVYKVLK	
1587,8893	1587,8809	-0,0084	-5	40	52	VYVYKVLKQVHPD	
2176,1648	2176,1699	0,0051	2	40	58	VYVYKVLKQVHPDTGISSK	Lys->AminoadipicAcid (K)[19]
1487,7893	1487,7563	-0,0330	-22	41	52	YVYKVLKQVHPD	Lys->Allysine (K)[4]
1894,9910	1894,9987	0,0077	4	41	56	YVYKVLKQVHPDTGIS	Oxidation (K)[4], Oxidation (Y)[1,3]
1325,7576	1325,7316	-0,0260	-20	42	52	VYKVLKQVHPD	
1683,9429	1683,9345	-0,0084	-5	42	56	VYKVLKQVHPDTGIS	
1770,9749	1770,9687	-0,0062	-4	42	57	VYKVLKQVHPDTGISS	
1899,0698	1899,0901	0,0203	11	42	58	VYKVLKQVHPDTGISSK	
2101,1475	2101,1194	-0,0281	-13	42	60	VYKVLKQVHPDTGISSKAM	
2603,3684	2603,3410	-0,0274	-11	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
2619,3633	2619,3280	-0,0353	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
2619,3633	2619,3280	-0,0353	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (M)[22]
2619,3633	2619,3280	-0,0353	-13	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (K)[17]
2750,4368	2750,4047	-0,0321	-12	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	
2766,4316	2766,3927	-0,0389	-14	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[25]
2766,4316	2766,3927	-0,0389	-14	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[17]
2766,4316	2766,3927	-0,0389	-14	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	Oxidation (P)[10]
1800,0015	1799,9834	-0,0181	-10	43	58	YKVLKQVHPDTGISSK	
2341,2366	2341,2270	-0,0096	-4	44	65	KVLKQVHPDTGISSKAMGIMNS	
2488,3049	2488,2877	-0,0172	-7	44	66	KVLKQVHPDTGISSKAMGIMNSF	
1293,7162	1293,7202	0,0040	3	45	56	VLKQVHPDTGIS	

1508,8431	1508,8411	-0,0020	-1	45	58	VLKQVHPDTGISSK	
2213,1416	2213,0964	-0,0452	-20	45	65	VLKQVHPDTGISSKAMGIMNS	
2360,2100	2360,2165	0,0065	3	45	66	VLKQVHPDTGISSKAMGIMNSF	
2376,2051	2376,1906	-0,0145	-6	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (F)[22]
2376,2051	2376,1906	-0,0145	-6	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2148,0576	2147,9975	-0,0601	-28	47	66	KQVHPDTGISSKAMGIMNSF	
2164,0525	2163,9902	-0,0623	-29	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (F)[20]
2164,0525	2163,9902	-0,0623	-29	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (M)[17]
2164,0525	2163,9902	-0,0623	-29	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (K)[12]
1744,8357	1744,7993	-0,0364	-21	49	65	VHPDTGISSKAMGIMNS	
1891,9041	1891,8857	-0,0184	-10	49	66	VHPDTGISSKAMGIMNSF	
1443,6970	1443,6844	-0,0126	-9	53	66	TGISSKAMGIMNSF	
1459,6919	1459,6832	-0,0087	-6	53	66	TGISSKAMGIMNSF	Oxidation (M)[8]
1085,5118	1085,5205	0,0087	8	57	66	SKAMGIMNSF	
998,4797	998,5010	0,0213	21	58	66	KAMGIMNSF	
870,3848	870,4079	0,0231	27	59	66	AMGIMNSF	
889,3937	889,3929	-0,0008	-1	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]
1126,5527	1126,5669	0,0142	13	65	73	SFVNDIFER	
1039,5208	1039,5409	0,0201	19	66	73	FVNDIFER	
892,4523	892,4754	0,0231	26	67	73	VNDIFER	
934,4992	934,5044	0,0052	6	70	77	IFERIAGE	
1248,6694	1248,6787	0,0093	7	70	80	IFERIAGEASR	
1278,6437	1278,6577	0,0140	11	70	80	IFERIAGEASR	Carbonyl (G)[7], Oxidation (R)[4]
1135,5854	1135,5996	0,0142	13	71	80	FERIAGEASR	
1215,6705	1215,6884	0,0179	15	78	87	ASRLAHYNKR	
1990,0101	1989,9934	-0,0167	-8	78	94	ASRLAHYNKRSTITSRE	Arg->GluSA (R)[10], Carbamyl (R)[16]
1990,0577	1990,0934	0,0357	18	78	94	ASRLAHYNKRSTITSRE	
1675,8875	1675,8770	-0,0105	-6	81	94	LAHYNKRSTITSRE	
1845,8824	1845,8781	-0,0043	-2	81	94	LAHYNKRSTITSRE	His->Asn (H)[3], Lys->AminoadipicAcid (K)[6], Oxidation (Y)[4]

1751,0425	1751,0330	-0,0095	-5	94	109	EIQTAVRLLLPGELAK	
2274,2815	2274,2197	-0,0618	-27	94	114	EIQTAVRLLLPGELAKHAVSE	
1622,0000	1621,9854	-0,0146	-9	95	109	IQTAVRLLLPGELAK	
1279,8096	1279,8346	0,0250	20	98	109	AVRLLLPGELAK	
896,5563	896,5681	0,0118	13	99	106	VRLLLPGE	
1009,6404	1009,6344	-0,0060	-6	99	107	VRLLLPGEL	
1080,6775	1080,6827	0,0052	5	99	108	VRLLLPGELA	
1208,7725	1208,7903	0,0178	15	99	109	VRLLLPGELAK	
1515,9369	1515,9312	-0,0057	-4	99	112	VRLLLPGELAKHAV	
1730,9800	1731,0188	0,0388	22	99	114	VRLLLPGELAKHAVSE	Lys->Allysine (K)[11]
1732,0116	1731,9855	-0,0261	-15	99	114	VRLLLPGELAKHAVSE	
797,4879	797,5053	0,0174	22	100	106	RLLLPGE	
1109,7041	1109,7149	0,0108	10	100	109	RLLLPGELAK	
1416,8685	1416,8883	0,0198	14	100	112	RLLLPGELAKHAV	
1631,9115	1631,8865	-0,0250	-15	100	114	RLLLPGELAKHAVSE	Lys->Allysine (K)[10]
1632,9431	1632,9417	-0,0014	-1	100	114	RLLLPGELAKHAVSE	
1794,9960	1794,9895	-0,0065	-4	100	114	RLLLPGELAKHAVSE	Glycation (K)[10]
1660,9017	1660,9186	0,0169	10	100	114	RLLLPGELAKHAVSE	Carbonyl (G)[6], Pro->pyro-Glu (P)[5]
1476,8420	1476,8372	-0,0048	-3	101	114	LLLPGELAKHAVSE	
2176,1860	2176,1496	-0,0364	-17	101	121	LLLPGELAKHAVSEGTKAVTK	Lys->Allysine (K)[9,17], Oxidation (HW)[10]
2176,1860	2176,1496	-0,0364	-17	101	121	LLLPGELAKHAVSEGTKAVTK	Lys->Allysine (K)[17], Lys->AminoadipicAcid (K)[9]
1363,7579	1363,7412	-0,0167	-12	102	114	LLPGELAKHAVSE	
1472,7227	1472,7178	-0,0049	-3	103	116	LPGELAKHAVSEGT	Oxidation (LV)[1,5], Oxidation (LV)[10], Oxidation (K)[7]
1753,8966	1753,9303	0,0337	19	103	119	LPGELAKHAVSEGTKAV	Oxidation (LV)[1,5], Oxidation (LV)[10], Lys->Allysine (K)[15]
1416,6853	1416,6959	0,0106	7	104	117	PGELAKHAVSEGTK	His->Asp (H)[7], Lys->AminoadipicAcid (K)[14]
1486,7748	1486,7670	-0,0078	-5	113	126	SEGTKAVTKYTSSK	

	1269,6686	1269,6896	0,0210	17	115	126	GTKAVTKYTSSK	Lys->Allysine (K)[12]
	1270,7002	1270,7007	0,0005	0	115	126	GTKAVTKYTSSK	
	1432,7529	1432,7481	-0,0048	-3	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H2B	1278,6688	1278,6952	0,0264	21	2	13	PEPAKSAPAPKK	Pro->pyro-Glu (P)[8,10], Proglutamicsealde (P)[3]
Ox_2 - 24h	1934,0706	1934,0742	0,0036	2	2	20	PEPAKSAPAPKKGSKKAVT	Carbamyl (K)[15], Lys->Allysine (K)[16]
	1450,7271	1450,6943	-0,0328	-23	6	16	KSAPAPKKGSK	Glycation (K)[7,8], Pro->pyro-Glu (P)[4,6]
	875,4145	875,4166	0,0021	2	35	41	KESYSVY	
	1137,5463	1137,5697	0,0234	21	35	43	KESYSVYVY	
	1265,6412	1265,6417	0,0005	0	35	44	KESYSVYVYK	
	1008,5036	1008,5139	0,0103	10	37	44	SYSVYVYK	
	1220,6561	1220,6556	-0,0005	0	37	46	SYSVYVYKVL	
	2120,1387	2120,1579	0,0192	9	39	57	SVYVYKVLKQVHPDTGISS	
	2248,2336	2248,2353	0,0017	1	39	58	SVYVYKVLKQVHPDTGISSK	
	2161,2017	2161,1803	-0,0214	-10	40	58	VYVYKVLKQVHPDTGISSK	
	1894,9910	1894,9891	-0,0019	-1	41	56	YVYKVLKQVHPDTGIS	Oxidation (K)[4], Oxidation (Y)[1,3]
	1325,7576	1325,7432	-0,0144	-11	42	52	VYKVLKQVHPD	
	1683,9429	1683,9403	-0,0026	-2	42	56	VYKVLKQVHPDTGIS	
	1770,9749	1770,9816	0,0067	4	42	57	VYKVLKQVHPDTGISS	
	1899,0698	1899,0893	0,0195	10	42	58	VYKVLKQVHPDTGISSK	
	2101,1475	2101,0969	-0,0506	-24	42	60	VYKVLKQVHPDTGISSKAM	
	2158,1689	2158,1737	0,0048	2	42	61	VYKVLKQVHPDTGISSKAMG	
	2619,3633	2619,3378	-0,0255	-10	42	65	VYKVLKQVHPDTGISSKAMGIMNS	Oxidation (N)[23]
	2603,3684	2603,3329	-0,0355	-14	42	65	VYKVLKQVHPDTGISSKAMGIMNS	
	2750,4368	2750,4149	-0,0219	-8	42	66	VYKVLKQVHPDTGISSKAMGIMNSF	

1800,0015	1799,9543	-0,0472	-26	43	58	YKVLKQVHPDTGISSK	
1421,8112	1421,8086	-0,0026	-2	44	56	KVLKQVHPDTGIS	
1636,9381	1636,9278	-0,0103	-6	44	58	KVLKQVHPDTGISSK	
1293,7162	1293,7092	-0,0070	-5	45	56	VLKQVHPDTGIS	
1380,7482	1380,7295	-0,0187	-14	45	57	VLKQVHPDTGISS	
1508,8431	1508,8177	-0,0254	-17	45	58	VLKQVHPDTGISSK	
1507,8115	1507,7999	-0,0116	-8	45	58	VLKQVHPDTGISSK	Lys->Allysine (K)[14]
1710,9207	1710,9583	0,0376	22	45	60	VLKQVHPDTGISSKAM	
1767,9421	1767,8992	-0,0429	-24	45	61	VLKQVHPDTGISSKAMG	
2012,0667	2012,0758	0,0091	5	45	63	VLKQVHPDTGISSKAMGIM	
2213,1416	2213,1171	-0,0245	-11	45	65	VLKQVHPDTGISSKAMGIMNS	
2376,2051	2376,1742	-0,0309	-13	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (N)[20]
2376,2051	2376,1742	-0,0309	-13	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (M)[19]
2376,2051	2376,1742	-0,0309	-13	45	66	VLKQVHPDTGISSKAMGIMNSF	Oxidation (K)[14]
2360,2100	2360,1999	-0,0101	-4	45	66	VLKQVHPDTGISSKAMGIMNSF	
1799,9142	1799,8685	-0,0457	-25	47	63	KQVHPDTGISSKAMGIM	
2000,9893	2000,9415	-0,0478	-24	47	65	KQVHPDTGISSKAMGIMNS	
2164,0525	2164,0617	0,0092	4	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (M)[14]
2164,0525	2164,0617	0,0092	4	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (F)[20]
2164,0525	2164,0617	0,0092	4	47	66	KQVHPDTGISSKAMGIMNSF	Oxidation (K)[12]
2148,0576	2148,0532	-0,0044	-2	47	66	KQVHPDTGISSKAMGIMNSF	
1744,8357	1744,8484	0,0127	7	49	65	VHPDTGISSKAMGIMNS	
1907,8990	1907,8478	-0,0512	-27	49	66	VHPDTGISSKAMGIMNSF	Oxidation (F)[18]
1907,8990	1907,8478	-0,0512	-27	49	66	VHPDTGISSKAMGIMNSF	Oxidation (M)[15]
1891,9041	1891,9100	0,0059	3	49	66	VHPDTGISSKAMGIMNSF	
1443,6970	1443,6838	-0,0132	-9	53	66	TGISSKAMGIMNSF	
1085,5118	1085,5363	0,0245	23	57	66	SKAMGIMNSF	
886,3797	886,3899	0,0102	12	59	66	AMGIMNSF	Oxidation (M)[5]
886,3797	886,3899	0,0102	12	59	66	AMGIMNSF	Oxidation (N)[6]
870,3848	870,4058	0,0210	24	59	66	AMGIMNSF	
889,3937	889,3839	-0,0098	-11	65	71	SFVNDIF	Oxidation (N)[4], Oxidation (F)[2,7]

754,3770	754,3843	0,0073	10	66	71	FVNDIF	
1039,5208	1039,5159	-0,0049	-5	66	73	FVNDIFER	
892,4523	892,4650	0,0127	14	67	73	VNDIFER	
1262,6376	1262,6644	0,0268	21	67	77	VNDIFERIAGE	
934,4992	934,5049	0,0057	6	70	77	IFERIAGE	
1248,6694	1248,6786	0,0092	7	70	80	IFERIAGEASR	
1135,5854	1135,6005	0,0151	13	71	80	FERIAGEASR	
1675,8875	1675,8517	-0,0358	-21	81	94	LAHYNKRSTITSRE	
1751,0425	1751,0281	-0,0144	-8	94	109	EIQTAVRLLLPGELAK	
2274,2815	2274,2319	-0,0496	-22	94	114	EIQTAVRLLLPGELAKHAVSE	
1309,7838	1309,8049	0,0211	16	95	106	IQTAVRLLLPGE	
1622,0000	1621,9921	-0,0079	-5	95	109	IQTAVRLLLPGELAK	
1919,1073	1919,0926	-0,0147	-8	96	113	QTAVRLLLPGELAKHAVS	Oxidation (P)[9]
1919,1072	1919,0926	-0,0146	-8	96	113	QTAVRLLLPGELAKHAVS	Oxidation (L)[8]
1380,8573	1380,8838	0,0265	19	97	109	TAVRLLLPGELAK	
1151,7146	1151,7410	0,0264	23	98	108	AVRLLLPGELA	
1279,8096	1279,8439	0,0343	27	98	109	AVRLLLPGELAK	
1278,7780	1278,7952	0,0172	13	98	109	AVRLLLPGELAK	Lys->Allysine (K)[12]
1803,0487	1803,0248	-0,0239	-13	98	114	AVRLLLPGELAKHAVSE	
1037,5990	1037,6197	0,0207	20	99	107	VRLLLPGEL	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1236,7311	1236,7252	-0,0059	-5	99	109	VRLLLPGELAK	Carbonyl (G)[7], Pro->pyro-Glu (P)[6]
1207,7408	1207,7620	0,0212	18	99	109	VRLLLPGELAK	Lys->Allysine (K)[11]
1515,9369	1515,9469	0,0100	7	99	112	VRLLLPGELAKHAV	
1737,0269	1737,0164	-0,0105	-6	99	113	VRLLLPGELAKHAVS	Glycation (K)[11], Pro->Pyrrolidone (P)[6]
1894,0643	1894,0197	-0,0446	-24	99	114	VRLLLPGELAKHAVSE	Glycation (K)[11]
1732,0116	1731,9950	-0,0166	-10	99	114	VRLLLPGELAKHAVSE	
1730,9800	1730,9786	-0,0014	-1	99	114	VRLLLPGELAKHAVSE	Lys->Allysine (K)[11]
797,4879	797,5071	0,0192	24	100	106	RLLLPGE	
981,6091	981,6006	-0,0085	-9	100	108	RLLLPGELA	
1137,6627	1137,6598	-0,0029	-3	100	109	RLLLPGELAK	Carbonyl (G)[6], Pro->pyro-Glu (P)[5]

1109,7041	1109,7242	0,0201	18	100	109	RLLLPGELAK	
1108,6725	1108,7030	0,0305	28	100	109	RLLLPGELAK	Lys->Allysine (K)[10]
1632,9431	1632,9449	0,0018	1	100	114	RLLLPGELAKHAVSE	
1631,9115	1631,9031	-0,0084	-5	100	114	RLLLPGELAKHAVSE	Lys->Allysine (K)[10]
1476,8420	1476,8204	-0,0216	-15	101	114	LLLPGELAKHAVSE	
1363,7579	1363,7540	-0,0039	-3	102	114	LLPGELAKHAVSE	
1486,7748	1486,7561	-0,0187	-13	113	126	SEGTKAVTKYTSSK	
1270,7002	1270,7175	0,0173	14	115	126	GTKAVTKYTSSK	
1432,7529	1432,7477	-0,0052	-4	115	126	GTKAVTKYTSSK	Glycation (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1903,9923	-0,0200	-11	2	21	SETAPAAPAAAPPAEKTPVK	
Control	3010,7158	3010,6597	-0,0561	-19	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	1276,7260	1276,7339	0,0079	6	9	21	PAAAPPAEKTPVK	
	966,5618	966,5755	0,0137	14	13	21	PPAEKTPVK	
	1326,7627	1326,7676	0,0049	4	34	46	KASGPPVSELITK	
	1340,7421	1340,7474	0,0053	4	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	845,5090	845,5264	0,0174	21	55	63	SGVSLAALK	
	973,6040	973,6096	0,0056	6	55	64	SGVSLAALKK	
	1706,8820	1706,8792	-0,0028	-2	64	79	KALAAAGYDVEKNNSR	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1903,9904	-0,0219	-12	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 30min	3010,7158	3010,6697	-0,0461	-15	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	2215,3396	2215,3318	-0,0078	-4	11	32	AAPPAEKTPVKKKAAKKPAGAR	
	966,5618	966,5765	0,0147	15	13	21	PPAEKTPVK	
	1326,7627	1326,7596	-0,0031	-2	34	46	KASGPPVSELITK	
	1340,7421	1340,7506	0,0085	6	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1340,7419	1340,7506	0,0087	6	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	845,509	845,5296	0,0206	24	55	63	SGVSLAALK	
	973,604	973,6145	0,0105	11	55	64	SGVSLAALKK	
	1578,787	1578,7832	-0,0038	-2	65	79	ALAAAGYDVEKNNSR	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1904,0010	-0,0113	-6	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 1h	2387,2566	2387,2748	0,0182	8	2	25	SETAPAAPAAAPPAEKTPVKKKAA	Carbamyl (K)[20,21], Lys->Allysine (K)[22]
	3010,7158	3010,7810	0,0652	22	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	3052,6538	3052,6800	0,0262	9	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[8,12,13]
	1326,7627	1326,7723	0,0096	7	34	46	KASGPPVSELITK	
	1326,7627	1326,7501	-0,0126	-9	34	46	KASGPPVSELITK	
	1340,7419	1340,7603	0,0184	14	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1340,7421	1340,7603	0,0182	14	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1198,6677	1198,6479	-0,0198	-17	35	46	ASGPPVSELITK	
	1212,6471	1212,6646	0,0175	14	35	46	ASGPPVSELITK	Carbonyl (G)[3]
	845,509	845,5081	-0,0009	-1	55	63	SGVSLAALK	
	973,604	973,6251	0,0211	22	55	64	SGVSLAALKK	
	1578,787	1578,7893	0,0023	1	65	79	ALAAAGYDVEKNNSR	
	1913,2157	1913,2317	0,0160	8	80	97	IKLGLKSLVSKGTLVQTK	
	1978,3011	1978,3170	0,0159	8	169	187	KVAKSPKKAKAAKPKKAAK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1903,9967	-0,0156	-8	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 3h	2387,2566	2387,2616	0,0050	2	2	25	SETAPAAPAAAPPAEKTPVKKKAA	Carbamyl (K)[20,21], Lys->Allysine (K)[22]
	3074,7319	3074,6859	-0,0460	-15	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Dioxidation (P)[12], Pro->Pyrrolidone (P)[13], Proglutamicsealde (P)[5,8]
	3074,6956	3074,6859	-0,0097	-3	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Oxidation (P)[5,8,12,13]
	3010,7158	3010,6338	-0,0820	-27	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	

3069,7056	3069,7629	0,0573	19	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Lys->Allysine (K)[22], Proglutamicsealde (P)[5,8]
3010,7158	3010,7793	0,0635	21	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
3052,6538	3052,6759	0,0221	7	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[8,12,13]
1276,7260	1276,7206	-0,0054	-4	9	21	PAAAPPAEKTPVK	
2215,3396	2215,3755	0,0359	16	11	32	AAPPAEKTPVKKKAAKKPAGAR	
2144,3025	2144,2759	-0,0266	-12	12	32	APPAEKTPVKKKAAKKPAGAR	
966,5618	966,5576	-0,0042	-4	13	21	PPAEKTPVK	
1326,7627	1326,7828	0,0201	15	34	46	KASGPPVSELITK	
1340,7421	1340,7422	0,0001	0	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1340,7419	1340,7422	0,0003	0	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1198,6678	1198,6929	0,0251	21	35	46	ASGPPVSELITK	Pro->Pyrrolidinone (P)[5], Proglutamicsealde (P)[4]
1198,6677	1198,6929	0,0252	21	35	46	ASGPPVSELITK	
1212,6471	1212,6631	0,0160	13	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6631	0,0160	13	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
845,509	845,5102	0,0012	1	55	63	SGVSLAALK	
973,604	973,6111	0,0071	7	55	64	SGVSLAALKK	
1335,6539	1335,6259	-0,0280	-21	65	77	ALAAAGYDVEKNN	
1185,7928	1185,7682	-0,0246	-21	80	90	IKLGLKSLVSK	
1913,2157	1913,2202	0,0045	2	80	97	IKLGLKSLVSKGTLVQTK	
1970,2372	1970,2230	-0,0142	-7	80	98	IKLGLKSLVSKGTLVQTKG	
2356,3669	2356,3800	0,0131	6	122	146	KAGAAKPKKAAGAAKKTKKATGAAT	Lys->Allysine (K)[16], Oxidation (K)[18,19]
2562,4727	2562,5006	0,0279	11	122	148	KAGAAKPKKAAGAAKKTKKATGAATPK	Carbonyl (G)[22], Lys->Allysine (K)[18,19]
2700,5479	2700,5561	0,0082	3	122	148	KAGAAKPKKAAGAAKKTKKATGAATPK	Carbamyl (K)[9,15], Oxidation (K)[8,16,18,19]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1904,0034	-0,0089	-5	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 24h	2387,2566	2387,2594	0,0028	1	2	25	SETAPAAPAAAPPAEKTPVKKKAA	Carbamyl (K)[20,21], Lys->Allysine (K)[22]
	3010,7158	3010,7642	0,0484	16	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[8,12,13]
	3052,6538	3052,6834	0,0296	10	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	2794,6414	2794,6882	0,0468	17	4	32	TAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	2215,3396	2215,3696	0,0300	14	11	32	AAPPAEKTPVKKKAAKKPAGAR	
	966,5618	966,5615	-0,0003	0	13	21	PPAEKTPVK	
	1326,7627	1326,7511	-0,0116	-9	34	46	KASGPPVSELITK	
	1340,7421	1340,7612	0,0191	14	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1340,7419	1340,7612	0,0193	14	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1198,6677	1198,6469	-0,0208	-17	35	46	ASGPPVSELITK	
	1212,6471	1212,6584	0,0113	9	35	46	ASGPPVSELITK	Carbonyl (G)[3]
	1212,6471	1212,6584	0,0113	9	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
	845,5090	845,4999	-0,0091	-11	55	63	SGVSLAALK	
	973,6040	973,5786	-0,0254	-26	55	64	SGVSLAALKK	
	1578,787	1578,7903	0,0033	2	65	79	ALAAAGYDVEKNNSR	
	1185,7928	1185,7638	-0,0290	-24	80	90	IKLGLKSLVSK	
	1913,2157	1913,2152	-0,0005	0	80	97	IKLGLKSLVSKGTLVQTK	
	1970,2372	1970,2349	-0,0023	-1	80	98	IKLGLKSLVSKGTLVQTKG	
	2562,4727	2562,4662	-0,0065	-3	122	148	KAGAAKPKKAAGAAKKTKKATGAATPK	Carbonyl (G)[22], Lys->Allysine (K)[19,27]
	2562,4250	2562,4126	-0,0124	-5	122	148	KAGAAKPKKAAGAAKKTKKATGAATPK	Lys->Allysine (K)[18,19,27], Lys->AminoadipicAcid (K)[16]
	1978,3011	1978,2920	-0,0091	-5	169	187	KVAKSPKKAKAAKPKKAAK	
	2335,5022	2335,5237	0,0215	9	169	191	KVAKSPKKAKAAKPKKAAKSAAK	

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	3066,6331	3066,6851	0,0520	17	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[5,8,12,13]
Ox_2 - 30min	2794,6050	2794,5891	-0,0159	-6	4	32	TAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Oxidation (P)[11], Pro->pyro-Glu (P)[10], Pro->Pyrrolidinone (P)[16]
	2794,6414	2794,5891	-0,0523	-19	4	32	TAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	2794,5798	2794,5891	0,0093	3	6	32	PAAPAAAPPAEKTPVKKKAAKKPAGAR	Carbamyl (K)[12,16,17,18]
	966,5618	966,5840	0,0222	23	13	21	PPAEKTPVK	
	1326,7627	1326,7705	0,0078	6	34	46	KASGPPVSELITK	
	1340,7421	1340,7387	-0,0034	-3	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1340,7419	1340,7387	-0,0032	-2	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1728,9495	1728,9421	-0,0074	-4	48	64	VAASKERSGVSLAALKK	Oxidation (V)[10], Lys->Allysine (K)[16,17]
	845,5090	845,5106	0,0016	2	55	63	SGVSLAALK	
	973,6040	973,6274	0,0234	24	55	64	SGVSLAALKK	
	2913,8205	2913,8277	0,0072	2	178	205	KAAKPKKAAKSAAKAVKPKAAKPKVAKP	Carbamyl (K)[7,10], Oxidation (K)[24]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1904,0043	-0,0080	-4	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_2 - 1h	3010,7158	3010,6416	-0,0742	-25	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	3052,6538	3052,6886	0,0348	11	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[8,12,13]
	3051,6433	3051,6939	0,0506	17	3	32	ETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Dioxidation (P)[4,7,11,12]
	3034,7007	3034,7003	-0,0004	0	6	33	PAAPAAAPPAEKTPVKKKAAKKPAGARR	Dioxidation (P)[4,8], Proglutamicsealde (P)[23]
	966,5618	966,5583	-0,0035	-4	13	21	PPAEKTPVK	
	2403,4055	2403,3886	-0,0169	-7	19	40	PVKKKAAKKPAGARRKASGPPV	Carbamyl (K)[4,5], Carbamyl (R)[14], Oxidation (K)[16], Oxidation (R)[15]

1326,7627	1326,7640	0,0013	1	34	46	KASGPPVSELITK	
1340,7419	1340,7388	-0,0031	-2	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7388	-0,0033	-2	34	46	KASGPPVSELITK	Carbonyl (G)[4]
2042,1133	2042,1197	0,0064	3	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19], Oxidation (K)[1]
1999,1075	1999,1100	0,0025	1	34	53	KASGPPVSELITKAVAASKE	Oxidation (V)[7]
1999,1075	1999,1100	0,0025	1	34	53	KASGPPVSELITKAVAASKE	Oxidation (P)[6]
1198,6677	1198,6451	-0,0226	-19	35	46	ASGPPVSELITK	
1212,6471	1212,6573	0,0102	8	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6573	0,0102	8	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
1898,0234	1898,0299	0,0065	3	35	53	ASGPPVSELITKAVAASKE	Carbamyl (K)[18]
1855,0176	1855,0231	0,0055	3	35	53	ASGPPVSELITKAVAASKE	
1826,9863	1826,9924	0,0061	3	36	53	SGPPVSELITKAVAASKE	Carbamyl (K)[17]
1783,9805	1783,9984	0,0179	10	36	53	SGPPVSELITKAVAASKE	
1739,9543	1739,9532	-0,0011	-1	37	53	GPPVSELITKAVAASKE	Carbamyl (K)[16]
1696,9485	1696,9515	0,0030	2	37	53	GPPVSELITKAVAASKE	
1682,9328	1682,9404	0,0076	5	38	53	PPVSELITKAVAASKE	Carbamyl (K)[15]
1639,9270	1639,9229	-0,0041	-3	38	53	PPVSELITKAVAASKE	
1715,0179	1715,0201	0,0022	1	48	65	VAASKERSGVSLAALKK	
845,5090	845,5090	0,0000	0	55	63	SGVSLAALK	
973,6040	973,6257	0,0217	22	55	64	SGVSLAALKK	
1335,6539	1335,6262	-0,0277	-21	65	77	ALAAAGYDVEKNN	
1185,7928	1185,7882	-0,0046	-4	80	90	IKLGLKSLVSK	
1913,2157	1913,2174	0,0017	1	80	97	IKLGLKSLVSKGTLVQTK	
1970,2372	1970,2208	-0,0164	-8	80	98	IKLGLKSLVSKGTLVQTKG	
2142,2862	2142,2884	0,0022	1	80	100	IKLGLKSLVSKGTLVQTKGTG	Carbonyl (G)[19]
2128,3069	2128,2979	-0,0090	-4	80	100	IKLGLKSLVSKGTLVQTKGTG	
2343,3970	2343,4185	0,0215	9	80	102	IKLGLKSLVSKGTLVQTKGTGAS	
2286,3760	2286,3692	-0,0068	-3	80	105	IKLGLKSLVSKGTLVQTKGTGASGSF	
2335,5022	2335,5093	0,0071	3	169	191	KVAKSPKKAKAAKPKKAAKSAAK	
2643,6513	2643,6709	0,0196	7	176	201	KAKAAKPKKAAKSAAKAVKPKAAKPK	Pro->pyro-Glu (P)[7,20]

	2673,7347	2673,7298	-0,0049	-2	181	206	KPKKAAKSAAKAVKPKAAKPKVAKPK	Dioxidation (P)[20], Pro->Pyrrolidone (P)[15]
	2453,5335	2453,5417	0,0082	3	191	213	KAVKPKAAKPKVAKPKKAAPKKK	Lys->Allysine (K)[11,14], Lys->AminoadipicAcid (K)[16]
	2585,6458	2585,6711	0,0253	10	191	213	KAVKPKAAKPKVAKPKKAAPKKK	Carbamyl (K)[6,9,11], Oxidation (K)[4]
	2129,4014	2129,3652	-0,0362	-17	193	212	VKPKAAKPKVAKPKKAAPKK	Pro->pyro-Glu (P)[12], Pro->Pyrrolidone (P)[2], Proglutamicsealde (P)[7]
	2241,5014	2241,4902	-0,0112	-5	193	213	VKPKAAKPKVAKPKKAAPKKK	
	1976,1908	1976,1493	-0,0415	-21	194	211	KPKAAKPKVAKPKKAAPK	Lys->AminoadipicAcid (K)[8], Proglutamicsealde (P)[7]
	1929,2489	1929,2450	-0,0039	-2	194	211	KPKAAKPKVAKPKKAAPK	Carbamyl (K)[8]
	1902,2380	1902,2266	-0,0114	-6	194	211	KPKAAKPKVAKPKKAAPK	Oxidation (P)[7]
	1902,2380	1902,2266	-0,0114	-6	194	211	KPKAAKPKVAKPKKAAPK	Oxidation (K)[8]
	2158,4279	2158,3998	-0,0281	-13	194	213	KPKAAKPKVAKPKKAAPKKK	Lys->Allysine (K)[11], Lys->AminoadipicAcid (K)[8]
	2185,4388	2185,4279	-0,0109	-5	194	213	KPKAAKPKVAKPKKAAPKKK	Carbamyl (K)[6]
	2156,4122	2156,3993	-0,0129	-6	194	213	KPKAAKPKVAKPKKAAPKKK	Pro->pyro-Glu (P)[7]
	1661,0954	1661,1020	0,0066	4	196	211	KAAKPKVAKPKKAAPK	
	1718,1532	1718,1687	0,0155	9	198	213	AKPKVAKPKKAAPKKK	
	1662,0794	1662,0611	-0,0183	-11	199	213	KPKVAKPKKAAPKKK	Lys->AminoadipicAcid (K)[3]
	1690,1219	1690,1388	0,0169	10	199	213	KPKVAKPKKAAPKKK	Carbamyl (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1903,9978	-0,0145	-8	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_2 - 3h	2160,2021	2160,2087	0,0066	3	2	23	SETAPAAPAAAPPAEKTPVKKK	
	2387,2566	2387,2608	0,0042	2	2	25	SETAPAAPAAAPPAEKTPVKKKAA	Carbamyl (K)[20,21], Lys->Allysine (K)[22]

3010,7158	3010,7520	0,0362	12	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
3010,7161	3010,7520	0,0359	12	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->Pyrrolidinone (P)[13], Proglutamicsealde (P)[12]
3052,6538	3052,6759	0,0221	7	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[8,12,13]
3069,7056	3069,7698	0,0642	21	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Lys->Allysine (K)[21], Proglutamicsealde (P)[5,8]
2215,3396	2215,3787	0,0391	18	11	32	AAPPAEKTPVKKKAAKKPAGAR	
966,5618	966,5879	0,0261	27	13	21	PPAEKTPVK	
1326,7627	1326,7621	-0,0006	0	34	46	KASGPPVSELITK	
1340,7419	1340,7644	0,0225	17	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7644	0,0223	17	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1198,6677	1198,6475	-0,0202	-17	35	46	ASGPPVSELITK	
1898,0234	1897,9987	-0,0247	-13	35	53	ASGPPVSELITKAVAASKE	Carbamyl (K)[18]
1855,0176	1855,0046	-0,0130	-7	35	53	ASGPPVSELITKAVAASKE	
1826,9864	1826,9645	-0,0219	-12	36	53	SGPPVSELITKAVAASKE	Carbamyl (K)[17]
1783,9806	1783,9699	-0,0107	-6	36	53	SGPPVSELITKAVAASKE	
1739,9543	1739,9334	-0,0209	-12	37	53	GPPVSELITKAVAASKE	Carbamyl (K)[16]
1696,9485	1696,9383	-0,0102	-6	37	53	GPPVSELITKAVAASKE	
1639,9270	1639,9188	-0,0082	-5	38	53	PPVSELITKAVAASKE	
1715,0179	1715,0042	-0,0137	-8	48	64	VAASKERSGVSLAALKK	
845,5090	845,5045	-0,0045	-5	55	63	SGVSLAALK	
973,6040	973,6290	0,0250	26	55	64	SGVSLAALKK	
1185,7928	1185,7882	-0,0046	-4	80	90	IKLGLKSLVSK	
1913,2157	1913,2131	-0,0026	-1	80	97	IKLGLKSLVSKGTLVQTK	
1970,2372	1970,2208	-0,0164	-8	80	98	IKLGLKSLVSKGTLVQTKG	
2142,2862	2142,2434	-0,0428	-20	80	100	IKLGLKSLVSKGTLVQTKGTG	Carbonyl (G)[19]
2128,3069	2128,3005	-0,0064	-3	80	100	IKLGLKSLVSKGTLVQTKGTG	
2286,3760	2286,3623	-0,0137	-6	80	102	IKLGLKSLVSKGTLVQTKGTGAS	
2629,6720	2629,6404	-0,0316	-12	176	201	KAKAAKPKKAAKSAAKAVKPKAAKPK	Pro->pyro-Glu (P)[7,20]

	2589,4139	2589,3906	-0,0233	-9	178	202	KAAKPKKAAKSAAKAVKPKAAKPKV	Oxidation (LV)[25], Lys->Allysine (K)[14,17], Lys->AminoadipicAcid (K)[7,10,19,22]
	2589,4139	2589,3906	-0,0233	-9	178	202	KAAKPKKAAKSAAKAVKPKAAKPKV	Lys->Allysine (K)[14,17], Lys->AminoadipicAcid (K)[7,10,19,22], Oxidation (K)[24]
	2453,5335	2453,5237	-0,0098	-4	191	213	KAVKPKAAKPKVAKPKKAAPKKK	Lys->Allysine (K)[12,15], Lys->AminoadipicAcid (K)[17]
	2584,6380	2584,6122	-0,0258	-10	194	213	KAVKPKAAKPKVAKPKKAAPKKK	Carbamyl (K)[6,8,11], Oxidation (K)[3]
	2199,3704	2199,3286	-0,0418	-19	194	213	KPKAAKPKVAKPKKAAPKKK	Carbamyl (K)[6], Lys->Allysine (K)[8], Lys->AminoadipicAcid (K)[3]
	2156,3646	2156,3430	-0,0216	-10	194	213	KPKAAKPKVAKPKKAAPKKK	Lys->Allysine (K)[8], Lys->AminoadipicAcid (K)[6]
	2185,4388	2185,4301	-0,0087	-4	194	213	KPKAAKPKVAKPKKAAPKKK	Carbamyl (K)[6]
	2156,4123	2156,3907	-0,0216	-10	194	213	KPKAAKPKVAKPKKAAPKKK	Pro->pyro-Glu (P)[7]
	1661,0954	1661,1004	0,0050	3	196	211	KAAKPKVAKPKKAAPK	
	2241,0000	2240,9574	-0,0426	-19	196	213	VKPKAAKPKVAKPKKAAPKKK	
	1718,1532	1718,1549	0,0017	1	198	213	AKPKVAKPKKAAPKKK	
	1662,0793	1662,1092	0,0299	18	199	213	KPKVAKPKKAAPKKK	Lys->AminoadipicAcid (K)[8]
	1690,1219	1690,1236	0,0017	1	199	213	KPKVAKPKKAAPKKK	Carbamyl (K)[3]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
native H1	1904,0123	1903,9917	-0,0206	-11	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_2 - 24h	2387,2566	2387,2667	0,0101	4	2	25	SETAPAAPAAAPPAEKTPVKKKAA	Carbamyl (K)[20,21], Lys->Allysine (K)[22]
	3010,7158	3010,7502	0,0344	11	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	
	3052,6538	3052,6859	0,0321	11	2	32	SETAPAAPAAAPPAEKTPVKKKAAKKPAGAR	Pro->pyro-Glu (P)[8,12,13]

2171,1819	2171,1781	-0,0038	-2	4	25	TAPAAPAAAPPAEKTPVKKKAA	Carbamyl (K)[19,20], Lys->Allysine (K)[18]
2215,3396	2215,3577	0,0181	8	11	32	AAPPAEKTPVKKKAAKKPAGAR	
1326,7627	1326,7483	-0,0144	-11	34	46	KASGPPVSELITK	
1340,7419	1340,7645	0,0226	17	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7645	0,0224	17	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1397,7998	1397,7926	-0,0072	-5	34	47	KASGPPVSELITKA	
2026,1184	2026,0860	-0,0324	-16	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19]
1983,1126	1983,0908	-0,0218	-11	34	53	KASGPPVSELITKAVAASKE	
1198,6677	1198,6464	-0,0213	-18	35	46	ASGPPVSELITK	
1212,6471	1212,6608	0,0137	11	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1855,0176	1854,9990	-0,0186	-10	35	53	ASGPPVSELITKAVAASKE	
1715,0179	1715,0230	0,0051	3	48	64	VAASKERSGVSLAALKK	
845,509	845,4944	-0,0146	-17	55	63	SGVSLAALK	
973,604	973,5806	-0,0234	-24	55	64	SGVSLAALKK	
1185,7928	1185,7694	-0,0234	-20	80	90	IKLGLKSLVSK	
1785,1207	1785,0925	-0,0282	-16	80	96	IKLGLKSLVSKGTLVQT	
1913,2157	1913,2196	0,0039	2	80	97	IKLGLKSLVSKGTLVQTK	
1970,2372	1970,235	-0,0022	-1	80	98	IKLGLKSLVSKGTLVQTKG	
2071,2849	2071,2959	0,0110	5	80	99	IKLGLKSLVSKGTLVQTKGT	
2128,3069	2128,3026	-0,0043	-2	80	100	IKLGLKSLVSKGTLVQTKGTG	
2157,2494	2157,2192	-0,0302	-14	80	100	IKLGLKSLVSKGTLVQTKGTG	Carbonyl (G)[19], Lys->AminoadipicAcid (K)[18]
2199,344	2199,3638	0,0198	9	80	101	IKLGLKSLVSKGTLVQTKGTGA	
2286,376	2286,3600	-0,0160	-7	80	102	IKLGLKSLVSKGTLVQTKGTGAS	
3430,4295	3430,4055	-0,0240	-7	80	104	IKLGLKSLVSKGTLVQTKGTGASGS	
2591,4772	2591,4306	-0,0466	-18	80	105	IKLGLKSLVSKGTLVQTKGTGASGSF	Carbonyl (G)[19]
2577,4979	2577,4695	-0,0284	-11	80	105	IKLGLKSLVSKGTLVQTKGTGASGSF	
1777,9659	1777,9837	0,0178	10	86	104	SLVSKGTLVQTKGTGASGS	
2548,5408	2548,5962	0,0554	22	110	136	KAATGEAKPKAKKAGAAKPKKAAGAAK	

2562,425	2562,484	0,0590	23	122	148	KAGAAKPKKAAGAAKKTKKATGAATPK	Lys->Allysine (K)[15,18,19], Lys->AminoadipicAcid (K)[16]
2562,4727	2562,484	0,0113	4	122	148	KAGAAKPKKAAGAAKKTKKATGAATPK	Carbonyl (G)[12], Lys->Allysine (K)[18,19]
2335,5022	2335,5093	0,0071	3	168	191	KVAKSPKKAKAAKPKKAAKSAAK	
2133,3285	2133,3093	-0,0192	-9	184	205	KAAKSAAKAVKPKAAKPKVAK	Carbamyl (K)[8], Lys->Allysine (K)[18]
1661,0954	1661,0954	0,0000	0	196	211	KAAKPKVAKPKKAAPK	
1704,0059	1703,9735	-0,0324	-19	196	211	KAAKPKVAKPKKAAPK	Lys->Allysine (K)[9,11], Lys->AminoadipicAcid (K)[16], Proglutamicsealde (P)[15]
1832,1961	1832,1741	-0,0220	-12	196	212	KAAKPKVAKPKKAAPKK	Carbamyl (K)[6,9]
1932,2485	1932,2176	-0,0309	-16	196	213	KAAKPKVAKPKKAAPKKK	Lys->AminoadipicAcid (K)[4]
1960,2911	1960,2931	0,0020	1	196	213	KAAKPKVAKPKKAAPKKK	Carbamyl (K)[6]
1718,1532	1718,1498	-0,0034	-2	198	213	AKPKVAKPKKAAPKKK	
1690,1219	1690,1185	-0,0034	-2	199	213	KPKVAKPKKAAPKKK	Carbamyl (K)[3]
1533,0004	1532,9774	-0,0230	-15	200	213	PKVAKPKKAAPKKK	Pro->pyro-Glu (P)[1]
1519,0212	1519,0349	0,0137	9	200	213	PKVAKPKKAAPKKK	
1491,0262	1491,0501	0,0239	16	200	213	PKVAKPKKAAPKKK	Pro->Pyrrolidone (P)[1]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1946,1069	1946,1093	0,0024	1	5	24	APAAPAAAPPAEKTPVKKKA	Oxidation (P)[9,10]
Control	2487,3076	2487,3401	0,0325	13	5	25	APAAPAAAPPAEKTPVKKKAA	Glycation (K)[17,18,19], Oxidation (P)[10]
	2545,4824	2545,4105	-0,0719	-28	9	32	PAAAPPAEKTPVKKKAAKKPAGAR	Glycation (K)[19]
	1326,7627	1326,7739	0,0112	8	34	46	KASGPPVSELITK	
	1198,6677	1198,6656	-0,0021	-2	35	46	ASGPPVSELITK	
	973,6040	973,6129	0,0089	9	55	64	SGVSLAALKK	
	1578,7870	1578,7922	0,0052	3	65	79	ALAAAGYDVEKNNSR	
	1520,9006	1520,9149	0,0143	9	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]
	1719,0379	1719,0308	-0,0072	-4	149	162	KTAKKTPKKAKKPA	Glycation (K)[9], Oxidation (K)[8,12]
	1972,1801	1972,1338	-0,0463	-23	161	178	PAAAAVTKKVAKSPKKAK	Glycation (K)[15], Oxidation (K)[16]
	735,4611	735,4622	0,0011	2	168	172	KKVAK	Glycation (K)[5]
	1520,9159	1520,9249	0,0090	6	181	195	KPKKAAKSAAKAVKP	Lys->Allysine (K)[7,14]
	2342,4022	2342,4287	0,0265	11	182	201	PKKAAKSAAKAVKPKAAKPK	Glycation (K)[18,20]
	1513,9320	1513,9228	-0,0092	-6	191	203	KAVKPKAAKPKVA	Glycation (K)[11], Oxidation (K)[4]
	1513,9320	1513,9228	-0,0092	-6	191	203	KAVKPKAAKPKVA	Glycation (K)[11], Oxidation (P)[5]
	804,4830	804,4822	-0,0008	-1	196	201	KAAKPK	Glycation (K)[4]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1520,7228	1520,7511	0,0283	19	4	19	TAPAAPAAAPPAEKTP	Dioxidation (P)[16], Pro->pyro-Glu (P)[10,11]
Ox_1 - 30min	2487,3076	2487,3401	0,0325	13	5	25	APAAPAAAPPAEKTPVKKKAA	Glycation (K)[17,18,19], Oxidation (P)[10]
	2515,4128	2515,4232	0,0104	4	5	28	APAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[23], Lys->AminoadipicAcid (K)[22]

	2544,3659	2544,3135	-0,0524	-21	6	26	PAAPAAAPPAEKTPVKKKAAK	Glycation (K)[16,17,18], Oxidation (K)[21]
	2108,1960	2108,1515	-0,0445	-21	9	27	PAAAPPAEKTPVKKKAAKK	Glycation (K)[19], Lys->Allysine (K)[9], Oxidation (K)[18]
	1326,7627	1326,7699	0,0072	5	34	46	KASGPPVSELITK	
	1340,7421	1340,7509	0,0088	7	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1340,7419	1340,7509	0,0090	7	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1384,6954	1384,7083	0,0129	9	34	46	KASGPPVSELITK	Carbonyl (G)[4], Oxidation (K)[1], Pro->pyro-Glu (P)[5,6]
	1198,6677	1198,6676	-0,0001	0	35	46	ASGPPVSELITK	
	1578,7870	1578,7897	0,0027	2	65	79	ALAAAGYDVEKNNSR	
	1520,9006	1520,8911	-0,0095	-6	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]
	1505,8929	1505,9094	0,0165	11	174	187	PKKAKAAKPKKAAK	Glycation (K)[2], Carbamyl (K)[8], Lys->Allysine (K)[10,11,14]
	1667,0584	1667,0417	-0,0166	-10	200	213	PKVAKPKKAAPKKK	Glycation (K)[2], Pro->pyro-Glu (P)[6], Pro->Pyrrolidone (P)[1]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1904,0123	1903,9757	-0,0366	-19	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 1h	2328,2068	2328,2232	0,0164	7	5	23	APAAPAAAPPAEKTPVKKK	Glycation (K)[17,18,19], Lys->Allysine (K)[13]
	2603,4402	2603,4164	-0,0238	-9	5	29	APAAPAAAPPAEKTPVKKKAAKKPA	Glycation (K)[18], Dioxidation (P)[15], Pro->pyro-Glu (P)[10,24], Pro->Pyrrolidone (P)[9]
	2236,1594	2236,1128	-0,0466	-21	6	25	PAAPAAAPPAEKTPVKKKAA	Glycation (K)[16,17], Lys->Allysine (K)[12,18]
	2236,2546	2236,2128	-0,0418	-19	6	26	PAAPAAAPPAEKTPVKKKAAK	Glycation (K)[18], Oxidation (K)[12,21]
	2429,4126	2429,3743	-0,0383	-16	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[17]

3226,8745	3226,8833	0,0088	3	6	36	PAAPAAAPPAEKTPVKKKAAKKPAGARRKAS	Glycation (K)[22]
2631,4939	2631,4816	-0,0123	-5	9	32	PAAAPPAEKTPVKKKAAKKPAGAR	Glycation (K)[19], Carbamyl (K)[13,18]
2707,5352	2707,5177	-0,0175	-6	9	32	PAAAPPAEKTPVKKKAAKKPAGAR	Glycation (K)[15,19]
1946,0593	1946,0057	-0,0536	-28	13	31	PPAEKTPVKKKAAKKPAGA	Lys->Allysine (K)[14], Lys->AminoadipicAcid (K)[10,11]
1610,8273	1610,8034	-0,0239	-15	14	27	PAEKTPVKKKAAKK	Carbamyl (K)[9], Lys->Allysine (K)[10,13], Lys->AminoadipicAcid (K)[8,14], Oxidation (K)[4]
1326,7627	1326,7439	-0,0188	-14	34	46	KASGPPVSELITK	
1326,7628	1326,7705	0,0077	6	34	46	KASGPPVSELITK	Pro->Pyrrolidinone (P)[6], Proglutamicsealde (P)[5]
1340,7419	1340,7755	0,0336	25	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7755	0,0334	25	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1354,7213	1354,7457	0,0244	18	34	46	KASGPPVSELITK	Carbonyl (G)[4], Pro->pyro-Glu (P)[5]
2026,1184	2026,0900	-0,0284	-14	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19]
1983,1126	1983,0967	-0,0159	-8	34	53	KASGPPVSELITKAVAASKE	
1997,0919	1997,0400	-0,0519	-26	34	53	KASGPPVSELITKAVAASKE	Carbonyl (G)[4]
1198,6677	1198,6455	-0,0222	-19	35	46	ASGPPVSELITK	
1212,6471	1212,6659	0,0188	16	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6659	0,0188	16	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
1756,9921	1756,9517	-0,0404	-23	48	64	VAASKERSGVSLAALKK	Carbamyl (K)[16], Lys->Allysine (K)[17]
845,5090	845,5030	-0,0060	-7	55	63	SGVSLAALK	
973,6040	973,5950	-0,0090	-9	55	64	SGVSLAALKK	
1031,5731	1031,5758	0,0027	3	55	64	SGVSLAALKK	Carbamyl (K)[10], Lys->AminoadipicAcid (K)[9]
1107,5681	1107,5630	-0,0051	-5	65	75	ALAAAGYDVEK	
1578,7870	1578,7854	-0,0016	-1	65	79	ALAAAGYDVEKNNSR	
1740,8398	1740,8259	-0,0139	-8	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
1513,7862	1513,7604	-0,0258	-17	92	106	TLVQTKGTGASGSFK	Dioxidation (F)[14]
1326,7017	1326,6980	-0,0037	-3	97	109	KGTGASGSFKLNK	Dioxidation (F)[9]
1520,9006	1520,8933	-0,0073	-5	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]

	1505,8929	1505,9015	0,0085	6	174	187	PKKAKAAKPKKAAK	Glycation (K)[2], Carbamyl (K)[14], Lys->Allysine (K)[8,10,11]
	1667,0584	1667,0570	-0,0013	-1	200	213	PKVAKPKKAAPKKK	Glycation (K)[2], Pro->pyro-Glu (P)[6], Pro->Pyrrolidone (P)[1]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1904,0123	1903,9954	-0,0169	-9	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 3h	1933,9866	1934,0081	0,0215	11	2	21	SETAPAAPAAAPPAEKTPVK	Oxidation (P)[18], Pro->pyro-Glu (P)[12], Pro->Pyrrolidinone (P)[13], Proglutamicsealde (P)[5]
	2004,1012	2004,1199	0,0187	9	5	23	APAAPAAAPPAEKTPVKKK	Glycation (K)[19], Lys->Allysine (K)[13]
	2458,2451	2458,2631	0,0180	7	5	24	APAAPAAAPPAEKTPVKKKA	Glycation (K)[17,18,19], Pro->pyro-Glu (P)[9,10], Proglutamicsealde (P)[15]
	2469,3445	2469,3235	-0,0210	-9	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,19], Oxidation (P)[9,10]
	2469,3445	2469,3235	-0,0210	-9	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,19], Oxidation (K)[13,22]
	2631,4351	2631,4313	-0,0039	-1	5	29	APAAPAAAPPAEKTPVKKKAAKKPA	Glycation (K)[18], Oxidation (K)[13,22], Pro->pyro-Glu (P)[10,15]
	2591,4653	2591,4447	-0,0206	-8	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[18,22]
	2631,5078	2631,4804	-0,0274	-10	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[16,18], Pro->Pyrrolidinone (P)[14,23], Pro->Pyrrolidone (P)[9]
	2544,4620	2544,4867	0,0247	10	9	33	PAAAPPAEKTPVKKKAAKKPAGARR	Arg->GluSA (R)[25], Oxidation (K)[18,19], Oxidation (R)[24]
	1945,1215	1945,0905	-0,0310	-16	13	27	PPAEKTPVKKKAAKK	Glycation (K)[11,15]
	2111,1936	2111,2359	0,0422	20	30	50	GARRKASGPPVSELITKAVAA	Oxidation (V)[11], Oxidation (P)[9]
	2111,1936	2111,2359	0,0422	20	30	50	GARRKASGPPVSELITKAVAA	Oxidation (K)[5,17]

1326,7627	1326,7786	0,0159	12	34	46	KASGPPVSELITK	
1340,7419	1340,7625	0,0206	15	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7625	0,0204	15	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1354,7213	1354,7285	0,0072	5	34	46	KASGPPVSELITK	Carbonyl (G)[4], Pro->pyro-Glu (P)[5]
1384,6954	1384,7009	0,0055	4	34	46	KASGPPVSELITK	Carbonyl (G)[4], Oxidation (K)[1], Pro->pyro-Glu (P)[5,6]
2026,1184	2026,0979	-0,0205	-10	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19]
1983,1126	1983,0848	-0,0278	-14	34	53	KASGPPVSELITKAVAASKE	
1198,6677	1198,6453	-0,0224	-19	35	46	ASGPPVSELITK	
1212,6471	1212,6201	-0,0270	-22	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6201	-0,0270	-22	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[5]
1212,6471	1212,6207	-0,0264	-22	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
845,5090	845,4946	-0,0144	-17	55	63	SGVSLAALK	
973,6040	973,5948	-0,0092	-9	55	64	SGVSLAALKK	
1031,5731	1031,5704	-0,0027	-3	55	64	SGVSLAALKK	Carbamyl (K)[10], Lys->AminoadipicAcid (K)[9]
1706,8820	1706,8564	-0,0256	-15	64	79	KALAAAGYDVEKNNSR	
1107,5681	1107,5553	-0,0128	-12	65	75	ALAAAGYDVEK	
1578,7870	1578,7871	0,0001	0	65	79	ALAAAGYDVEKNNSR	
1740,8398	1740,8158	-0,0240	-14	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
1513,7862	1513,7649	-0,0213	-14	92	105	TLVQTKGTGASGSFK	Dioxidation (F)[14]
1520,9006	1520,9118	0,0112	7	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]
1031,5369	1031,5404	0,0035	3	138	148	KKATGAATPK	Carbonyl (G)[5], Lys->AminoadipicAcid (K)[2], Proglutamicsealde (P)[9]
1198,6319	1198,6296	-0,0023	-2	142	153	TGAATPKKTAKK	Lys->Allysine (K)[7,11,12]
3282,8153	3282,7628	-0,0525	-16	161	191	PAAAAVTKKVAKSPKKAKAAKPKKAAKSAAK	Glycation (K)[15], Oxidation (V)[10], Lys->AminoadipicAcid (K)[12,16,18,21]
1495,9688	1495,9663	-0,0025	-2	191	204	KAVKPKAAKPKVAK	Oxidation (V)[12], Oxidation (P)[5]
1511,9637	1511,9810	0,0172	11	191	204	KAVKPKAAKPKVAK	Oxidation (K)[6,9,11]

	1080,7257	1080,7224	-0,0032	-3	199	208	KPKVAKPKKA	Oxidation (P)[2], Pro->Pyrrolidinone (P)[7]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1904,0123	1904,0309	0,0186	10	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_1 - 24h	3341,8049	3341,8621	0,0572	17	5	32	APAAPAAAPPAEKTPVKKKAAKKPAGAR	Glycation (K)[17,18,19,23]
	2429,4126	2429,3760	-0,0366	-15	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[22]
	1482,8639	1482,8687	0,0048	3	33	46	RKASGPPVSELITK	
	2284,2512	2284,2375	-0,0137	-6	33	54	RKASGPPVSELITKAVAASKER	Arg->GluSA (R)[1], Oxidation (K)[2,14]
	1326,7627	1326,7478	-0,0149	-11	34	46	KASGPPVSELITK	
	1340,7419	1340,7609	0,0190	14	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1340,7421	1340,7609	0,0188	14	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1354,7213	1354,7460	0,0247	18	34	46	KASGPPVSELITK	Carbonyl (G)[4], Pro->pyro-Glu (P)[5]
	1384,6954	1384,7037	0,0083	6	34	46	KASGPPVSELITK	Carbonyl (G)[4], Oxidation (K)[1], Pro->pyro-Glu (P)[5,6]
	2026,1184	2026,0860	-0,0324	-16	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19]
	1983,1126	1983,0928	-0,0198	-10	34	53	KASGPPVSELITKAVAASKE	
	1997,0919	1997,0939	0,0021	1	34	53	KASGPPVSELITKAVAASKE	Pro->pyro-Glu (P)[6]
	1997,0919	1997,0940	0,0021	1	34	53	KASGPPVSELITKAVAASKE	Carbonyl (G)[4]
	2031,0973	2031,1116	0,0142	7	34	53	KASGPPVSELITKAVAASKE	Oxidation (K)[1], Oxidation (P)[5,6]
	2031,0973	2031,1116	0,0142	7	34	53	KASGPPVSELITKAVAASKE	Dioxidation (P)[5], Oxidation (P)[6]
	1198,6677	1198,6536	-0,0141	-12	35	46	ASGPPVSELITK	
	1212,6471	1212,6641	0,0170	14	35	46	ASGPPVSELITK	Carbonyl (G)[3]
	1212,6471	1212,6641	0,0170	14	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
	1898,0234	1897,9930	-0,0304	-16	35	53	ASGPPVSELITKAVAASKE	Carbamyl (K)[18]
	1855,0176	1854,9990	-0,0186	-10	35	53	ASGPPVSELITKAVAASKE	
	2011,1188	2011,0786	-0,0402	-20	35	54	ASGPPVSELITKAVAASKER	
	1826,9863	1826,9680	-0,0183	-10	36	53	SGPPVSELITKAVAASKE	Carbamyl (K)[17]

1783,9805	1783,9734	-0,0071	-4	36	53	SGPPVSELITKAVAASKE	
997,5564	997,5458	-0,0106	-11	38	46	PPVSELITK	Pro->pyro-Glu (P)[2]
1796,0281	1795,9963	-0,0318	-18	38	54	PPVSELITKAVAASKER	
845,5090	845,5016	-0,0074	-9	55	63	SGVSLAALK	
973,6040	973,5926	-0,0114	-12	55	64	SGVSLAALKK	
1031,5731	1031,5759	0,0028	3	55	64	SGVSLAALKK	Carbamyl (K)[9], Lys->AminoadipicAcid (K)[10]
1706,8820	1706,8892	0,0072	4	64	79	KALAAAGYDVEKNNSR	
1107,5681	1107,5525	-0,0156	-14	65	75	ALAAAGYDVEK	
1578,7870	1578,7750	-0,0120	-8	65	79	ALAAAGYDVEKNNSR	
1740,8398	1740,8341	-0,0057	-3	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
1513,7862	1513,7604	-0,0258	-17	92	106	TLVQTKGTGASGSFK	Dioxidation (F)[14]
1520,9006	1520,9296	0,0290	19	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]
1242,6213	1242,6304	0,0091	7	138	149	TKKATGAATPKK	Carbonyl (G)[6], Lys->Allysine (K)[11,12], Proglutamicsealde (P)[10]
1505,8929	1505,9020	0,0091	6	174	187	PKKAKAAKPKKAAK	Glycation (K)[2], Carbamyl (K)[14], Lys->Allysine (K)[8,10,11]
1741,0111	1740,9850	-0,0261	-15	194	207	KPKAAKPKVAKPKK	Glycation (K)[6], Lys->AminoadipicAcid (K)[3,8], Proglutamicsealde (P)[2]
1758,9852	1759,0080	0,0228	13	195	207	PKAAKPKVAKPKK	Glycation (K)[5,7], Oxidation (K)[10], Pro->pyro-Glu (P)[6,11]
1759,0216	1759,0306	0,0090	5	195	207	PKAAKPKVAKPKK	Glycation (K)[5,7], Pro->pyro-Glu (P)[11], Proglutamicsealde (P)[6]
1665,1267	1665,1349	0,0082	5	199	213	KPKVAKPKKAAPKKK	Oxidation (P)[2], Pro->Pyrrolidone (P)[7], Proglutamicsealde (P)[12]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification

glycated H1	1520,7228	1520,7495	0,0267	18	4	19	TAPAAPAAAPPAEKTP	Dioxidation (P)[6], Pro->pyro-Glu (P)[10,11]
Ox_2 - 30min	2328,2068	2328,2232	0,0164	7	5	23	APAAPAAAPPAEKTPVKKK	Glycation (K)[17,18,19], Lys->Allysine (K)[13]
	2487,3076	2487,3401	0,0325	13	5	25	APAAPAAAPPAEKTPVKKKAA	Glycation (K)[17,18,19], Oxidation (P)[10]
	2603,4402	2603,4164	-0,0238	-9	5	29	APAAPAAAPPAEKTPVKKKAAKKPA	Glycation (K)[18], Dioxidation (P)[15], Pro->pyro-Glu (P)[10,24], Pro->Pyrrolidone (P)[9]
	3742,9596	3743,0307	0,0711	19	5	35	APAAPAAAPPAEKTPVKKKAAKKPAGARRKA	Glycation (K)[17,18,19], Lys->Allysine (K)[30], Lys->AminoadipicAcid (K)[13], Oxidation (K)[22,23]
	3434,9229	3434,9830	0,0601	17	5	39	APAAPAAAPPAEKTPVKKKAAKKPAGARRKASGPP	Arg->GluSA (R)[29], Carbamyl (K)[30], Oxidation (K)[13,22,23]
	3434,9343	3434,8830	-0,0513	-15	5	39	APAAPAAAPPAEKTPVKKKAAKKPAGARRKASGPP	Dioxidation (P)[24,34], Pro->pyro-Glu (P)[10], Pro->Pyrrolidinone (P)[15]
	2544,3659	2544,3135	-0,0524	-21	6	26	PAAPAAAPPAEKTPVKKKAAK	Glycation (K)[16,17,18], Oxidation (K)[21]
	3421,8061	3421,8604	0,0543	16	6	34	PAAPAAAPPAEKTPVKKKAAKKPAGARRK	Glycation (K)[22], Lys->Allysine (K)[12], Lys->AminoadipicAcid (K)[21,29]
	3434,8864	3434,8346	-0,0518	-15	6	38	PAAPAAAPPAEKTPVKKKAAKKPAGARRKASGP	Glycation (K)[22], Carbonyl (G)[25,32], Lys->Allysine (K)[21], Proglutamicsealde (P)[9]
	1946,0593	1946,0562	-0,0031	-2	13	31	PPAEKTPVKKKAAKKPAGA	Lys->Allysine (K)[14], Lys->AminoadipicAcid (K)[10,11]
	1326,7627	1326,7701	0,0074	6	34	46	KASGPPVSELITK	
	1340,7421	1340,7450	0,0029	2	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1340,7419	1340,7450	0,0031	2	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1384,6954	1384,7123	0,0169	12	34	46	KASGPPVSELITK	Carbonyl (G)[4], Oxidation (K)[1], Pro->pyro-Glu (P)[5,6]
	1198,6677	1198,6700	0,0023	2	35	46	ASGPPVSELITK	

	1212,6471	1212,6425	-0,0046	-4	35	46	ASGPPVSELITK	Carbonyl (G)[3]
	1578,7870	1578,7952	0,0082	5	65	79	ALAAAGYDVEKNNSR	
	1740,8398	1740,8158	-0,0240	-14	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
	1513,7862	1513,7649	-0,0213	-14	92	106	TLVQTKGTGASGSFK	Dioxidation (F)[14]
	1473,8528	1473,8692	0,0164	11	191	202	KAVKPKAAKPKV	Glycation (K)[9], Lys->AminoadipicAcid (K)[6], Oxidation (K)[4,11]
	1473,8528	1473,8692	0,0164	11	191	202	KAVKPKAAKPKV	Glycation (K)[9], Oxidation (V)[12], Lys->AminoadipicAcid (K)[6], Oxidation (K)[4]
	1933,1009	1933,1104	0,0095	5	195	211	PKAAKPKVAKPKKAAPK	Glycation (K)[7], Lys->Allysine (K)[5,10], Lys->AminoadipicAcid (K)[13]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1904,0123	1903,9942	-0,0181	-10	2	21	SETAPAAPAAAPPAEKTPVK	
Ox_2 - 1h	2469,3445	2469,2908	-0,0537	-22	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,19], Oxidation (P)[9,10]
	2469,3445	2469,2908	-0,0537	-22	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,18], Oxidation (K)[13,22]
	2603,4026	2603,3981	-0,0045	-2	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,18,19], Dioxidation (P)[9], Pro->Pyrrolidone (P)[10]
	3240,8176	3240,8727	0,0551	17	5	37	APAAPAAAPPAEKTPVKKKAAKKPAGARRKASG	Arg->GluSA (R)[29], Carbamyl (K)[13], Oxidation (K)[22,23,30]
	2545,3396	2545,3162	-0,0234	-9	6	29	PAAPAAAPPAEKTPVKKKAAKKPA	Glycation (K)[18], Lys->AminoadipicAcid (K)[16,17,21]
	2545,4712	2545,4662	-0,0050	-2	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[18], Oxidation (P)[23], Pro->Pyrrolidone (P)[9]

2603,4402	2603,4481	0,0079	3	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[22], Carbonyl (G)[25], Oxidation (P)[8,9]
2649,4458	2649,4535	0,0077	3	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[16], Oxidation (K)[12,17,21], Pro->pyro-Glu (P)[8], Proglutamicsealde (P)[9]
2649,5186	2649,5535	0,0349	13	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[16,17], Oxidation (P)[23], Pro->Pyrrolidinone (P)[8], Pro->Pyrrolidone (P)[9,14]
3226,8745	3226,7910	-0,0835	-26	6	36	PAAPAAAPPAEKTPVKKKAAKKPAGARRKAS	Glycation (K)[22]
2707,5352	2707,5146	-0,0206	-8	9	32	PAAAPPAEKTPVKKKAAKKPAGAR	Glycation (K)[15,19]
1610,8273	1610,8138	-0,0135	-8	14	27	PAEKTPVKKKAAKK	Carbamyl (K)[9], Lys->Allysine (K)[10,13], Lys->AminoadipicAcid (K)[8,14], Oxidation (K)[4]
1946,0930	1946,1000	0,0070	4	22	40	KKAAKKPAGARRKASGPPV	Carbonyl (G)[9], Lys->Allysine (K)[13], Lys->AminoadipicAcid (K)[2]
1482,8639	1482,8591	-0,0048	-3	33	46	RKASGPPVSELITK	
1326,7627	1326,7401	-0,0226	-17	34	46	KASGPPVSELITK	
1340,7419	1340,7338	-0,0081	-6	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7338	-0,0083	-6	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1354,7213	1354,7249	0,0036	3	34	46	KASGPPVSELITK	Carbonyl (G)[4], Pro->pyro-Glu (P)[5]
1354,7213	1354,7249	0,0036	3	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5,6]
1384,6954	1384,7018	0,0064	5	34	46	KASGPPVSELITK	Carbonyl (G)[4], Oxidation (K)[1], Pro->pyro-Glu (P)[5,6]
1397,7998	1397,7855	-0,0143	-10	34	47	KASGPPVSELITKA	
1198,6677	1198,6460	-0,0217	-18	35	46	ASGPPVSELITK	
1212,6471	1212,6631	0,0160	13	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6631	0,0160	13	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
1127,6306	1127,6213	-0,0093	-8	36	46	SGPPVSELITK	
845,5090	845,4984	-0,0106	-13	55	63	SGVSLAALK	
973,6040	973,5962	-0,0078	-8	55	64	SGVSLAALKK	

	1031,5731	1031,5592	-0,0139	-13	55	64	SGVSLAALKK	Carbamyl (K)[10], Lys->AminoadipicAcid (K)[9]
	1107,5681	1107,5520	-0,0161	-15	65	75	ALAAAGYDVEK	
	1578,7870	1578,7809	-0,0061	-4	65	79	ALAAAGYDVEKNNSR	
	1740,8398	1740,8048	-0,0350	-20	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
	1394,6659	1394,6700	0,0041	3	68	79	AAAGYDVEKNNSR	
	1513,7862	1513,7856	-0,0006	0	92	106	TLVQTKGTGASGSFK	Dioxidation (F)[14]
	1520,9006	1520,8993	-0,0013	-1	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]
	2171,1811	2171,2010	0,0199	9	148	166	KKTAKKTPKKAKKPAAAAV	Glycation (K)[10], Lys->Allysine (K)[9], Lys->AminoadipicAcid (K)[2,12,13]
	2431,4611	2431,4681	0,0070	3	149	169	KTAKKTPKKAKKPAAAAVTKK	Glycation (K)[9], Carbamyl (K)[11], Oxidation (K)[1,12]
	3431,9680	3431,9577	-0,0103	-3	161	191	PAAAAVTKKVAKSPKKAKAAKPKKAAKSAAK	Glycation (K)[12,15], Oxidation (V)[6,10], Lys->Allysine (K)[16], Oxidation (K)[9,18]
	1361,7998	1361,8097	0,0100	7	175	185	KKAKAAKPKKA	Glycation (K)[1], Lys->Allysine (K)[10], Oxidation (K)[4,7]
	1844,0016	1844,0311	0,0295	16	195	208	PKAAKPKVAKPKKA	Glycation (K)[5,7], Oxidation (V)[8], Pro->pyro-Glu (P)[1,6,11]
	1933,1009	1933,0959	-0,0050	-3	195	211	PKAAKPKVAKPKKAAPK	Glycation (K)[5], Lys->Allysine (K)[10,17], Lys->AminoadipicAcid (K)[2]
	1932,0693	1932,0789	0,0097	5	195	211	PKAAKPKVAKPKKAAPK	Glycation (K)[7], Lys->Allysine (K)[5,12,13], Lys->AminoadipicAcid (K)[10]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification

glycated H1	1520,7228	1520,7446	0,0218	14	4	19	TAPAAPAAAPPAEKTP	Dioxidation (P)[3], Pro->pyro-Glu (P)[11,16]
Ox_2 - 3h	2469,3445	2469,3108	-0,0337	-14	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,19], Oxidation (P)[9,10]
	2469,3445	2469,3108	-0,0337	-14	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,18], Oxidation (K)[13,22]
	2603,4026	2603,4181	0,0155	6	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,18,19], Dioxidation (P)[9], Pro->Pyrrolidone (P)[10]
	3272,7598	3272,8032	0,0434	13	5	37	APAAPAAAPPAEKTPVKKKAAKKPAGARRKASG	Arg->GluSA (R)[28,29], Carbamyl (K)[13,17], Oxidation (K)[18,19,22,23,30]
	2429,4126	2429,3806	-0,0320	-13	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[22]
	2591,4653	2591,4442	-0,0211	-8	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[18,22]
	2545,3396	2545,3064	-0,0332	-13	6	29	PAAPAAAPPAEKTPVKKKAAKKPA	Glycation (K)[18], Lys->AminoadipicAcid (K)[16,17,21]
	2487,3564	2487,3713	0,0149	6	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Carbonyl (G)[25], Oxidation (K)[12,16], Oxidation (P)[9,14], Pro->pyro-Glu (P)[23]
	2603,4402	2603,4355	-0,0047	-2	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[22], Carbonyl (G)[25], Oxidation (P)[8,9]
	2649,4458	2649,4396	-0,0062	-2	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[16], Oxidation (K)[12,17,21], Pro->pyro-Glu (P)[8], Proglutamicsealde (P)[9]
	1610,8273	1610,8155	-0,0118	-7	14	27	PAEKTPVKKKAAKK	Carbamyl (K)[9], Lys->Allysine (K)[10,13], Lys->AminoadipicAcid (K)[8,14], Oxidation (K)[4]
	1482,8639	1482,8682	0,0043	3	33	46	RKASGPPVSELITK	
	1326,7627	1326,7803	0,0176	13	34	46	KASGPPVSELITK	
	1340,7419	1340,7562	0,0143	11	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
	1340,7421	1340,7562	0,0141	11	34	46	KASGPPVSELITK	Carbonyl (G)[4]
	1354,7213	1354,7307	0,0094	7	34	46	KASGPPVSELITK	Carbonyl (G)[4], Pro->pyro-Glu (P)[5]

1354,7213	1354,7307	0,0094	7	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5,6]
2026,1184	2026,0900	-0,0284	-14	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19]
1983,1126	1983,0948	-0,0178	-9	34	53	KASGPPVSELITKAVAASKE	
1198,6677	1198,6450	-0,0227	-19	35	46	ASGPPVSELITK	
1212,6471	1212,6696	0,0225	19	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6696	0,0225	19	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
973,6040	973,5958	-0,0082	-8	55	64	SGVSLAALKK	
1578,7870	1578,7750	-0,0120	-8	65	79	ALAAAGYDVEKNNSR	
1740,8398	1740,8191	-0,0207	-12	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
1394,6659	1394,6589	-0,0070	-5	67	79	AAAGYDVEKNNSR	
1513,7862	1513,7856	-0,0006	0	92	106	TLVQTKGTGASGSFK	Dioxidation (F)[14]
1520,9006	1520,8906	-0,0100	-7	128	140	PKKAAGAAKKTKK	Glycation (K)[9], Oxidation (K)[3,10]
2534,4516	2534,4784	0,0267	11	128	151	PKKAAGAAKKTKKATGAATPKKTA	Glycation (K)[9], Oxidation (K)[3,10,12]
2398,2829	2398,3212	0,0384	16	129	152	KKAAGAAKKTKKATGAATPKKTAK	Glycation (K)[8], Lys->Allysine (K)[2,9], Lys->AminoadipicAcid (K)[1,20,24]
2171,1811	2171,1840	0,0029	1	148	166	KKTAKKTPKKAKKPAAAAV	Glycation (K)[10], Lys->Allysine (K)[9], Lys->AminoadipicAcid (K)[2,12,13]
823,4560	823,4698	0,0138	17	155	161	PKKAKKP	Lys->Allysine (K)[2,3,5], Proglutamicsealde (P)[1]
3431,9680	3431,9592	-0,0088	-3	161	191	PAAAAVTKKVAKSPKKAKAAKPKKAAKSAAK	Glycation (K)[12,15], Oxidation (V)[6,10], Lys->Allysine (K)[16], Oxidation (K)[9,18]
1361,7998	1361,8014	0,0016	1	175	185	KKAKAAKPKKA	Glycation (K)[1], Lys->Allysine (K)[10], Oxidation (K)[4,7]
1473,8528	1473,8601	0,0073	5	191	202	KAVKPKAAKPKV	Glycation (K)[9], Lys->AminoadipicAcid (K)[6], Oxidation (K)[4,11]
1473,8528	1473,8601	0,0073	5	191	202	KAVKPKAAKPKV	Glycation (K)[9], Oxidation (V)[12], Lys->AminoadipicAcid (K)[6], Oxidation (K)[4]

	1932,0693	1932,0662	-0,0031	-2	195	211	PKAAKPKVAKPKKAAPK	Glycation (K)[7], Lys->Allysine (K)[5,12,13], Lys->AminoadipicAcid (K)[10]

Sample	Calculated Mass	Observed Mass	Match Error Da	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Modification
glycated H1	1520,7228	1520,7363	0,0135	9	4	19	TAPAAPAAAPPAEKTP	Dioxidation (P)[3], Pro->pyro-Glu (P)[11,16]
Ox_2 - 24h	2469,3445	2469,3257	-0,0188	-8	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,19], Oxidation (P)[9,10]
	2469,3445	2469,3257	-0,0188	-8	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,18], Oxidation (K)[13,22]
	2603,4026	2603,4090	0,0064	2	5	26	APAAPAAAPPAEKTPVKKKAAK	Glycation (K)[17,18,19], Dioxidation (P)[9], Pro->Pyrrolidone (P)[10]
	2515,3992	2515,3774	-0,0218	-9	5	28	APAAPAAAPPAEKTPVKKKAAKKP	Carbamyl (K)[17,18,19], Oxidation (K)[13,22,23]
	2236,2546	2236,2486	-0,0060	-3	6	26	PAAPAAAPPAEKTPVKKKAAK	Glycation (K)[18], Oxidation (K)[13,21]
	2429,4126	2429,4115	-0,0011	0	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[17]
	2591,4653	2591,4772	0,0119	5	6	28	PAAPAAAPPAEKTPVKKKAAKKP	Glycation (K)[17,22]
	2487,3564	2487,3394	-0,0170	-7	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Carbonyl (G)[25], Oxidation (K)[12,16], Oxidation (P)[9,14], Pro->pyro-Glu (P)[23]
	2603,4402	2603,4545	0,0143	5	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[22], Carbonyl (G)[25], Oxidation (P)[8,9]
	2649,4458	2649,4216	-0,0242	-9	6	30	PAAPAAAPPAEKTPVKKKAAKKPAG	Glycation (K)[16], Oxidation (K)[12,17,21], Pro->pyro-Glu (P)[8], Proglutamicsealde (P)[9]
	3457,8647	3457,9502	0,0855	25	6	37	PAAPAAAPPAEKTPVKKKAAKKPAGARRKASG	Glycation (K)[16], Carbonyl (G)[32], Lys->Allysine (K)[17,18]

2707,5352	2707,5250	-0,0102	-4	9	32	PAAAPPAEKTPVKKKAAKKPAGAR	Glycation (K)[15,19]
1610,8273	1610,8171	-0,0102	-6	14	27	PAEKTPVKKKAAKK	Carbamyl (K)[9], Lys->Allysine (K)[10,13], Lys->AminoadipicAcid (K)[8,14], Oxidation (K)[4]
1482,8639	1482,8673	0,0034	2	33	46	RKASGPPVSELITK	
984,5359	984,5255	-0,0104	-11	34	43	KASGPPVSEL	
1326,7627	1326,7485	-0,0142	-11	34	46	KASGPPVSELITK	
1340,7419	1340,7678	0,0259	19	34	46	KASGPPVSELITK	Pro->pyro-Glu (P)[5]
1340,7421	1340,7678	0,0257	19	34	46	KASGPPVSELITK	Carbonyl (G)[4]
1397,7998	1397,7836	-0,0162	-12	34	47	KASGPPVSELITKA	
2026,1184	2026,1002	-0,0182	-9	34	53	KASGPPVSELITKAVAASKE	Carbamyl (K)[19]
1983,1126	1983,1047	-0,0079	-4	34	53	KASGPPVSELITKAVAASKE	
1997,0919	1997,0580	-0,0340	-17	34	53	KASGPPVSELITKAVAASKE	Carbonyl (G)[4]
1997,0919	1997,0579	-0,0340	-17	34	53	KASGPPVSELITKAVAASKE	Pro->pyro-Glu (P)[6]
1198,6677	1198,6361	-0,0316	-26	35	46	ASGPPVSELITK	
1212,6471	1212,6648	0,0177	15	35	46	ASGPPVSELITK	Carbonyl (G)[3]
1212,6471	1212,6648	0,0177	15	35	46	ASGPPVSELITK	Pro->pyro-Glu (P)[4]
1954,0609	1954,0519	-0,0090	-5	38	56	PPVSELITKAVAASKERSG	Pro->pyro-Glu (P)[1,2]
2126,1821	2126,1864	0,0043	2	38	58	PPVSELITKAVAASKERSGVS	
1060,6115	1060,6073	-0,0042	-4	45	54	TKAVAASKER	
1458,8280	1458,8280	0,0000	0	48	62	VAASKERSGVSLAAL	
973,6040	973,5994	-0,0046	-5	55	64	SGVSLAALKK	
1031,5731	1031,5762	0,0031	3	55	64	SGVSLAALKK	Carbamyl (K)[10], Lys->AminoadipicAcid (K)[9]
1578,7870	1578,7906	0,0036	2	65	79	ALAAAGYDVEKNNSR	
1740,8398	1740,8132	-0,0266	-15	65	79	ALAAAGYDVEKNNSR	Glycation (K)[11]
1394,6659	1394,6644	-0,0015	-1	67	79	AAAGYDVEKNNSR	
1513,7862	1513,8046	0,0184	12	92	106	TLVQTKGTGASGSFK	Dioxidation (F)[14]
2534,4516	2534,4660	0,0144	6	128	151	PKKAAGAAKKTKKATGAATPKKTA	Glycation (K)[9], Oxidation (K)[3,10,12]

2398,2829	2398,2595	-0,0234	-10	129	152	KKAAGAAKKTKKATGAATPKKTAK	Glycation (K)[8], Lys->Allysine (K)[2,9], Lys->AminoadipicAcid (K)[1,20,24]
1963,1188	1963,1345	0,0157	8	149	164	KTAKKTPKKAKKPAAA	Glycation (K)[9], Carbamyl (K)[11,12], Oxidation (K)[4,5,8]
2171,1811	2171,1799	-0,0012	-1	148	166	KKTAKKTPKKAKKPAAAAV	Glycation (K)[10], Lys->Allysine (K)[9], Lys->AminoadipicAcid (K)[2,12,13]
823,4560	823,4523	-0,0037	-4	155	161	PKKAKKP	Lys->Allysine (K)[2,3,5], Proglutamicsealde (P)[1]
3431,9680	3431,9691	0,0011	0	161	191	PAAAAVTKKVAKSPKKAKAAKPKKAAKSAAK	Glycation (K)[12,15], Oxidation (V)[6,10], Lys->Allysine (K)[16], Oxidation (K)[9,18]
1361,7998	1361,7910	-0,0088	-6	175	185	KKAKAAKPKKA	Glycation (K)[1], Lys->Allysine (K)[10], Oxidation (K)[4,7]
1473,8528	1473,8583	0,0055	4	191	202	KAVKPKAAKPKV	Glycation (K)[9], Lys->AminoadipicAcid (K)[6], Oxidation (K)[4,11]
1473,8528	1473,8583	0,0055	4	191	202	KAVKPKAAKPKV	Glycation (K)[9], Oxidation (V)[12], Lys->AminoadipicAcid (K)[6], Oxidation (K)[4]
1731,0267	1731,0171	-0,0096	-6	195	207	PKAAKPKVAKPKK	Glycation (K)[5,7], Pro->pyro-Glu (P)[11], Pro->Pyrrolidone (P)[1], Proglutamicsealde (P)[6]
1857,1060	1857,0731	-0,0329	-18	195	209	PKAAKPKVAKPKKAA	Glycation (K)[5,7]
1933,1009	1933,1401	0,0392	20	195	211	PKAAKPKVAKPKKAAPK	Glycation (K)[5], Lys->Allysine (K)[7,10], Lys->AminoadipicAcid (K)[13]